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Editorial

1  Biomedicine brings the future nearer
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Review Articles

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It is my greatest pleasure to introduce the inaugural issue of our journal “BioMedicine”. The goal of BioMedicine is to publish peer-reviewed, high-quality articles, especially in the field of translational and personalized medicine, thereby serving as a communication channel between peers and the wider public.

More and more outstanding scholars will share their scientific achievements with us and promote the progression of this journal.

We believe that this journal will provide updates and articles to advance our knowledge in the field of medicine, and will benefit surmounting medical obstacles in the future.

The resistance of cells and body to repetitive exposures to various stresses has ushered in a new scientific era of biomedicine. This issue features not only basic mechanisms, but also applications in clinical conditions. It has been divided into sections A and B. Section A, receptor biomedicine, is comprised of four papers and highlights the mechanisms of receptors. Paper 1 summarizes the current understanding and emerging impact of nuclear epidermal growth factor receptor (EGFR) pathways and how EGFR translocates into the nucleus in response to ionizing radiation, chemotherapy, and anti-EGFR target agents, in order to facilitate the development of novel strategies to overcome acquired resistance. Paper 2 indicates that NO can upregulate the expression and activity of Src. It also suggests that Src can mediate NF-κB activation, therefore, the authors perceived a loop of signal amplification to influence the inducible nitric oxide synthase (iNOS)/Src/focal adhesion kinase (FAK) axis for macrophage locomotion in response to engagement of Toll-like receptors. Paper 3 highlights the three known estrogen receptors (ERα, ERβ and GPR30). These confer cardioprotective effects against various stresses by preventing myocardial cell apoptosis and cardiac hypertrophy. Accumulating evidence reveals their roles in E2-mediated genomic and nongenomic pathways in cardiomyocytes against various cardiac insults: hypoxia, ischemic–reperfusion injury, sepsis, hypertrophic agents, plus other pro-apoptotic signals. Paper 4 points out that P2X7 receptor activation is a double-edged sword. Strong P2X7R activation-mediated neuron cell death is due to Ca²⁺ overload in the cytosol, and possible perturbation of Ca²⁺ homeostasis involved in the endoplasmic reticulum (ER) and mitochondria. By contrast, a putative role of basal or mild activity of P2X7R is known to exert anti-apoptotic or proliferative effect in cancer cells, glia and some neuroblastomas. P2X7R activation as a neurotoxic or neurotrophic signal is discussed.

These initial papers are followed by two papers pertain to regenerative biomedicine, highlighting the mechanisms of nerve regeneration and bone biomedical engineering. Paper 5 investigates the effects of electrical stimulation (ES) on peripheral nerve regeneration, using electrical treatment at different intervals, frequencies, and intensities to stimulate nerve segments in silicone rubber chambers. ES has two effects: (1) hindering the growth of regenerating nerves and (2) promoting their recovery. This reveals the importance of applying safe stimulus protocols. Incorrect use of ES could irreversibly damage nerve tissues and delay their regeneration. Paper 6 explains the repair of bone defects with gelatin-based composites. This paper shows that biomaterials, such as gelatin-based composites, are suitable for use as bone replacements, are biocompatible and can be reabsorbed or dissolve naturally as the bone grows, yielding newly remodeled bone. The genipin and Oligomeric proanthocyanidins (OPCs)-cross-linked gelatin/tricalcium phosphate (TCP) composite provides an emerging approach towards effective repair of damaged or diseased skeletal tissue.

In the first issue of BioMedicine, the aforementioned papers will be of great interest to biologists as well as clinicians dedicated to studying mechanisms and applications of receptor functions and regenerative biomedicine. Topics relating to traditional Chinese medicine (TCM) will be the highlight of the next issue of BioMedicine, and will supply valuable information and insight for researchers and investigators of multi-disciplines.

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Review article

Implication of nuclear EGFR in the development of resistance to anticancer therapies

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\begin{abstract}

Epidermal growth factor receptor (EGFR) was identified as a major oncogenic factor in various types of cancer, and thereby has been considered as an attractive therapeutic target for cancer therapy. The well-characterized classic function of this plasma membrane-bound receptor is transduction of extracellular mitogenic signals to a variety of intracellular downstream signaling cascades associated with tumorigenesis. Aberrantly expressed EGFR also undergoes direct nuclear translocation to induce transcription of genes associated with cell proliferation, cell cycle regulation, and tumor progression. Emerging evidence suggests the existence of a new role of nuclear EGFR signaling in conferring acquired resistance in response to various anticancer therapies. In this review, we summarize the current understanding of how EGFR translocates into the nucleus in response to ionizing radiation, chemotherapy, and anti-EGFR target agents. The emerging impact of nuclear EGFR in modulating the cellular sensitivity of cancer cells to these anticancer treatments will also be discussed. A better understanding of the nuclear EGFR pathways in response to anticancer therapies will facilitate the development of novel strategies to overcome the acquired resistance.

\end{abstract}

\section{Introduction}

Epidermal growth factor receptor (EGFR; ErbB1), a receptor tyrosine kinase, is frequently overexpressed and widely involved in the etiology and progression of many types of cancer \cite{1}. Cancer patients whose tumors aberrantly express EGFR tend to have a more aggressive disease and a shorter survival rate, so EGFR not only has been viewed as a predictive

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marker for poor clinical outcome but also intensely pursued as a therapeutic target [2]. EGFR activation through dimerization and autophosphorylation with ERBB family stimulates multiple intracellular downstream signaling pathways by recruiting effector proteins. Two major pathways initiated by the receptor tyrosine kinase are the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3 K)–Akt pathways [3,4]. Other important growth regulators in cancer in response to EGFR activation are the signal transducer and activator of transcription proteins (STATs), SRC tyrosine kinase, and mammalian target of rapamycin [5–7]. These signaling cascades integrate and transmit the EGFR activation into distinct transcriptional programs associated with proliferation, tumorigenesis, metastasis, and survival [3]. In addition to its well-characterized downstream signaling pathways, EGFR and other ErbB family have been found to enter the nucleus of multiple types of cancer cells and possess oncogenic functions, including gene transcription [8–12], DNA repair [13–15], regulation of enzyme activity [16], and translation [17], linking to the aggressiveness of tumors. Interestingly, the nuclear translocation of EGFR was also repeatedly observed in response to the constitutive treatment with different types of anticancer drugs [18–21] and ionizing radiation [14,22,23], suggesting that nuclear EGFR may play a crucial role in the development of therapy resistance. However, the role of nuclear EGFR in the development of resistance to anticancer therapies is still not fully understood. This review will summarize and discuss the current understanding of the nuclear functions of EGFR and its impact on tumor sensitivity to radiation, chemotherapy, and anti-EGFR target therapy.

2. Biological properties and clinical implication of nuclear EGFR in cancer

Following the first discovery of nuclear localization of EGFR in liver cancer cells [24], nuclear expression of EGFR and ErbB2 has been continually discovered in a variety of cancer types [8,10,25–31]. The first identified nuclear function of EGFR is regulation of gene transcription [10]. Although the association with AT-rich sequence (ATRS) of its target gene promoters has been proposed to be required for nuclear EGFR-activated gene transcription [10,21], the lack of a DNA-binding domain [10] suggests that nuclear EGFR targets promoters through binding to various transcriptional factors with DNA-binding domains and functions as a transcription cofactor rather than a DNA-binding transcription factor. In response to EGFR stimulation, the interaction of nuclear EGFR with signal transducer and activator of transcription-3 (STAT3) has been demonstrated to be required for nuclear EGFR-mediated inducible nitric oxide synthase (iNOS) [8] and cyclooxygenase-2 (COX-2) [9] expression. In addition, nuclear EGFR cooperates with STAT5 and E2F1 to enhance aurora A [32] and B-Myc [27] gene expression, respectively. Interestingly, our recent findings further revealed that nuclear EGFR also interacts with RNA helicase A (RHA) independent of its ATPase/helicase activity to associate with the promoter region of its target genes such as cyclin D1 and iNOS [33], indicating that RHA is a DNA-binding partner for nuclear EGFR in regulating its target gene expression. However, overexpression of the RHA-interaction domain (residues 645–1186) of EGFR is not sufficient and full length of EGFR is required to increase the promoter activity [33], suggesting that other unidentified components may be involved in the EGFR–RHA complex.

Given the fact that nuclear EGFR retains its tyrosine kinase activity, regulation of protein stability and enzymatic activity of its target proteins via tyrosine phosphorylation has been explored as another important nuclear function of EGFR. Proliferating cell nuclear antigen (PCNA) was the first identified nuclear substrate of EGFR tyrosine kinase [13]. Nuclear EGFR stabilized chromatin-bound PCNA protein via phosphorylating at its Tyr211 and preventing its polyubiquitination and proteasomal degradation. The increased PCNA Tyr211 phosphorylation by nuclear EGFR promotes cell proliferation and DNA repair, and is closely correlated with poor survival of breast cancer patients. In addition to targeting PCNA, nuclear EGFR was also found to enhance DNA repair via regulating DNA-dependent protein kinase (DNA–PK), an enzyme involved in repairing double-strand breaks and V(D)J recombination [34]. A substantial amount of DNA–PK was found to be colocalized with EGFR in anti-EGFR mAb-treated cells in the confocal microscope analysis [34]. The physical interaction between nuclear EGFR and DNA–PK was also observed in cancer cells treated with radiation [14] or anti-EGFR monoclonal antibody [34]. Furthermore, the nuclear level of EGFR is associated with phosphorylation of DNA–PK at residue T2609, an indicator of DNA–PK activity during nonhomologous end-joining DNA repair [14,22], and inhibition of EGFR signaling was accompanied by a reduction in the level and activity of DNA–PK in the nuclear fraction [34,35]. Although there is no evidence revealing that DNA–PK is directly phosphorylated by nuclear EGFR, these findings suggest that nuclear EGFR modulates DNA repair in response to DNA damage through regulating the kinase activity of the DNA–PK complex [36].

Although not all functions of nuclear EGFR have been elucidated, several studies suggest that nuclear EGFR may serve as a prognostic marker for poor clinical outcome. In a population of 130 breast cancer patients, tumor tissues from 37.7% of this cohort were immunostained positively for nuclear EGFR, and a significant inverse correlation existed between the high nuclear EGFR expression and overall survival [28]. Hadzisejdic et al also reported nuclear EGFR as an independent prognostic factor for mortality in another cohort of breast cancer patients [37]. The correlation between poor survival rate and high level of nuclear EGFR in the cancer cells was also observed in several cohorts of cancer patients with oral squamous carcinomas [28], oropharyngeal carcinomas [29], ovarian cancer [30], and esophageal squamous carcinomas [38]. These observations suggest that nuclear EGFR may be considered as a prognostic indicator for poor clinical outcome and also revealed a crucial role of nuclear EGFR in mediating tumor progression.

3. Nuclear EGFR facilitates the development of resistance to a variety of anticancer therapies

In most studies, nuclear localization of EGFR was observed in EGF-treated cancer cells or in the human primary tumor
tissues. EGF induces EGFR nuclear localization rapidly and transiently within 2 h of treatment [39]. Coat protein complex I-mediated retrograde trafficking from the Golgi to the endoplasmic reticulum (ER) has been shown to regulate EGF-induced EGFR nuclear transport [40]. In addition to these physiological situations, our and others’ recent studies uncovered that some anticancer therapies also drive EGFR import into the nucleus of various cancer cells, adding a role of nuclear EGFR in the development of drug resistance. Unlike the transient nuclear localization by EGF stimulation, EGFR is steadily present in the nucleus in response to these anticancer treatments [14,18–22,36].

Ionizing radiation (IR) was found to stimulate EGFR nuclear transport in human bronchial and squamous carcinoma cells [14,18,41]. Other DNA-damaging stimuli, such as cisplatin and H2O2 also initiated EGFR internalization and subsequent nuclear import [14,42]. In the nucleus, EGFR has been demonstrated to play important roles in unhooking cisplatin-induced interstrand crosslinks and in repairing IR-induced strand breaks, indicating the involvement of nuclear EGFR in confering chemoresistance and radioresistance [36]. Supporting this notion, our data further showed that after reconstruction of a functional nuclear localization sequence in its nuclear localization signal (NLS)-deleted mutant, EGFR is able to restore the DNA repair activity and consequently reduced the sensitivity of cancer cells to cisplatin [19].

In addition to IR and cisplatin, two EGFR-targeted therapeutic agents, cetuximab [41] and gefitinib [21], were also found to elicit the accumulation of EGFR in the nucleus. Cetuximab (Erbitux) is an EGFR-blocking antibody that has been approved for the treatment of patients with head and neck squamous cell carcinoma (HNSCC) and metastatic colorectal cancer. Ectopic expression of the NLS-tagged EGFR can reduce the sensitivity of NCI-H226 non-small cell lung cancer (NSCLC) cells to cetuximab both in vitro and in mouse xenografts [20], supporting the association of nuclear EGFR with tumor resistance to cetuximab. Gefitinib (ZD1839, Iressa), a small molecular weight EGFR kinase inhibitor, has been used for advanced and metastatic NSCLC with expression of activating EGFR mutants, such as EGFR L858R mutant [43], and most of NSCLC cancer patients bearing wild-type (wt) EGFR frequently are insensitive to this drug [43]. Our recent study reported that, in a wt EGFR-expressing cancer cell line, nuclear translocation of EGFR was increased in response to chronic treatment with gefitinib, and mediated the gene expression of breast cancer resistant protein (BCRP) to cause the development of drug resistance through efflux of gefitinib [21]. However, the nuclear translocation of EGFR and its mediated BCRP expression were only observed in cancer cells expressing wt EGFR but not its activating mutant, suggesting a possible mechanism explaining why gefitinib is not beneficial to most wt EGFR-expressing NSCLC patients [21,44]. The reason why the mutant EGFR lacks nuclear translocation ability is not clear. One possibility could be that the recently identified tracking mechanism of cell surface EGFR to the nucleus is impaired [40,45]. Similar to this observation, cells bearing EGFR L858R, which do not show nuclear expression, also possess less ability to repair cisplatin- and IR-induced DNA damage [36]. These studies imply a nuclear-specific role of wt EGFR in conferring the resistance in response to EGFR target therapy, chemotherapy, and IR.

EGFR tyrosine kinase activation has been reported to be required for the nuclear translocation of EGFR in response to EGF stimulation [10]. In addition to the wt form, EGFR is also present as truncated mutant (EGFRvIII) with constitutive kinase activity [46], and is associated with the aggressive biology of glioma [47]. The constitutively activated EGFR variant, EGFRvIII, is present in the nuclei of glial cells [48] and glioblastoma [48,49] revealing the crucial role of tyrosine kinase activation in the EGFR nuclear import. In response to irradiation, however, EGFR has been found to enter the nucleoplasm in a ligand-independent process that involves free radicals [14,50,51]. Furthermore, the L858R and exon 19 deletion mutants of EGFR, which exhibit constitutive kinase activity, did not show nuclear import after irradiation or cisplatin treatment [36], suggesting that other mechanisms, in addition to its kinase activity, may regulate the nuclear import of EGFR in response to these anticancer therapies. We have recently discussed the detailed mechanism by which the full-length receptors embedded in the endosomal membrane travel all the way from the cell surface to the early endosomes and pass through the nuclear pore complexes [40,45,52]. The specific regulations of EGFR nuclear import in response to different anticancer treatments will be described below.

4. Nuclear trafficking of EGFR in response to anticancer therapies

4.1. Ionizing radiation-induced EGFR nuclear localization involves karyopherin alpha and protein kinase C epsilon

Karyopherin proteins, also named importin, are nuclear transport factors and mediate the majority of nucleocytoplasmic transport [53]. We have demonstrated that interaction with importin beta is involved in the translocation of EGFR [39,45,54] and ErbB2 [55] into the nucleus through the nuclear pore complex. Mutation of its NLS disrupts the interaction of EGFR with importins, indicating that EGFR may interact with importins through its NLS motif [39,54]. Dittmann et al showed that ionizing radiation also enhanced nuclear EGFR to form a complex with karyopherin alpha and Ran protein, which are essential for formation of a nuclear localization sequence-dependent nuclear import complex [14]. Their work suggested that IR triggers EGFR import into the nucleus in a karyopherin alpha-linked manner. In parallel with the role of nuclear EGFR in repairing DNA damage, karyopherin has also been considered as a marker for global chemoresistance and been reported as an important factor of tumorigenesis and progression of breast cancer [56]. Interestingly, the interaction between EGFR and the alpha form of karyopherin was only observed in response to IR but not EGF stimulation [14], suggesting that interactions with karyopherin alpha and beta forms may be responsible for radiation- and EGF-induced EGFR nuclear translocation, respectively. It would also be of great interest to know what the different regulations for the EGFR interactions with various karyopherins are in response to these stimuli.
Phosphorylation of EGFR Thr654 by protein kinase C epsilon (PKCε) is another regulation for EGFR nuclear translocation following irradiation [23]. EGFR Thr654 phosphorylation has been reported earlier to block Cbl induced ubiquitination and lysosomal degradation of EGFR, leading to EGFR stabilization [57]. PKCε has been identified as the kinase responsible for this modification following irradiation [18]. Furthermore, deletion of Thr654 blocked nuclear transport of EGFR, whereas mutation to Glu increased this shuttling, demonstrating that phosphorylation of this residue is essential for nuclear EGFR shuttling following irradiation [23]. Because the Thr654 phosphorylation is located within the NLS motif of EGFR, it raises the possibility that this phosphorylation by PKCε may regulate EGFR interaction with karyopherins.

4.2 Phosphorylation by Src family kinases drives EGFR nuclear import to reduce the sensitivity to cetuximab

Cetuximab (C225, Erbitux), a humanized monoclonal antibody, recognizes the extracellular domain of both wt EGFR and EGFRvIII has been approved as the second-line treatment after failure to chemotherapy or as the first-line treatment with radiation therapy for advanced HNSCC. Cetuximab is also used in combination with irinotecan for treating metastatic colorectal cancer after failure to chemotherapy. Reduction of c-Cbl-mediated internalization and degradation of EGFR under the chronic exposure to cetuximab leads to steady-state expression of EGFR, and the increased EGFR confers cetuximab resistance through binding and activating HER2 or HER3 to maintain signaling to MAPK and Akt pathways [58]. In addition to activating other receptor tyrosine kinases, the increased EGFR expression also caused the accumulation of EGFR in the nucleus [20]. In contrast to the ligand-independent manner in the IR-treated cells, the nuclear translocation of EGFR in cetuximab-resistant cells seems to rely on overexpression of several ErbB family ligands, including EGF, amphiregulin, heparin-binding (HB) EGF and beta-cellulin [20]. Overexpression of these ligands enhances the nuclear translocation of EGFR through Src family kinases (SFKs), and treatment of cetuximab-resistant cells with SFK inhibitor dasatinib (BMS354825) can resensitize cells to cetuximab [20]. Because inhibition of SFK activity and EGFR Y845 phosphorylation by dasatinib resulted in loss of cetuximab-induced nuclear EGFR expression (Fig. 1) and increase in membrane

Fig. 1 – The nuclear roles of EGFR in conferring the resistance to irradiation, cisplatin, and anti-EGFR agents. In response to chronic treatment with irradiation and cisplatin, the nuclear translocation of EGFR is enhanced by PKCε and SFK-dependent phosphorylation at Thr654 and Tyr845, respectively, and by interaction with importins. The nuclear EGFR can interact with and activate DNA-PK to promote DNA repair, and thereby confers radioresistance and chemoresistance. In gefitinib-resistant cells, the compensatoryAkt activation by IGFIR signaling also facilitates EGFR nuclear import through phosphorylation of its Ser229. EGFR in the nucleus functions as a transcriptional regulator to mediate BCRP expression, which recognizes gefitinib and doxorubicin as substrates to result in the efflux of these drugs.
expression of EGF, Src may regulate the nuclear import of EGFR through phosphorylation of its Y845 and thereby contribute to cetuximab resistance [41].

4.3. Akt enhances wt EGFR nuclear import via phosphorylating its Ser229 response to gefitinib resistance

Gefitinib (ZD1839, Iressa) is the first small molecular inhibitor of EGFR tyrosine kinase. Although EGFR is overexpressed in many cancer types, targeting EGFR tyrosine kinase activity by gefitinib showed more dramatic efficacy and clinical benefits for NSCLC patients, particularly those characterized as East Asian, nonsmoker, adenocarcinoma histological type, and female gender, but only modest effects on many other cancer types [43]. The encouraging responses in these selected NSCLC patients to gefitinib showed strong association with specific gain-of-function mutations within the EGFR–tyrosine kinase domain [43,59]. Several mechanisms, including activation of c-MET and insulin growth factor receptor (IGFR) to raise the compensatory survival signals [60,61] and the loss of phosphatase and tensin homolog (PTEN) [62,63], have been shown to play a role in determining the sensitivity of wt EGFR-expressing cancer cell to gefitinib. Interestingly, these mechanisms commonly elevate the PI3K–Akt signaling pathway to maintain the cell survival in the presence of gefitinib [64,65]. In addition to providing the survival signaling, our recent study further disclosed that the elevated Akt activity was also associated with the nuclear localization of wt EGFR to mediate gefitinib resistance [21].

As in cetuximab-resistant cells [20] and irradiation-resistant cells [14,66], nuclear accumulation of EGFR in response to gefitinib resistance is observed in wt EGFR-expressing cell lines but not in EGFR mutant-expressing cell lines. We identified EGFR Ser229 as a novel Akt substrate and Ser229 phosphorylation of EGFR was detected in both nuclear and cytoplasmic fraction of gefitinib-resistant cells. Overexpression of Akt can dramatically increase the nuclear level of EGFR, and the Akt-mediated EGFR nuclear accumulation was attenuated by substitution of Ser229 to Ala, demonstrating that this phosphorylation is required for EGFR nuclear translocation [21]. Because elevated or continuous activation of Akt survival signaling is commonly observed in cancer cells with the characteristic of chemoresistance [19], radioresistance [14], or cetuximab insensitivity [20,58], Akt-dependent phosphorylation might be an general regulation for the nuclear translocation of EGFR in response to various anticancer therapies (as illustrated in Fig. 1). Currently, we further reported that sequential Akt-dependent phosphorylation and polyubiquitination are required for IκB kinase (IKKα) nuclear transportation in response to hepatitis B virus X protein overexpression [67]. Because ubiquitination has been widely found to be involved in protein nucleocyttoplasmic shuttling [68–71], it raises the possibility that polyubiquitination of EGFR also occurs following Akt-dependent phosphorylation and mediates EGFR nuclear import. These observations suggest that phosphorylation by Akt may function as a common signal to drive the nuclear trafficking of target proteins, including EGFR. Other regulations, such as polyubiquitination, involving interactions with nuclear importer or exporter might be required to decide the destination of these cargo proteins. However, to elucidate the detail mechanism further studies are needed.

5. Molecular actions of nuclear EGFR in the development of resistance to anticancer therapies

5.1. Nuclear EGFR regulates DNA repair involves DNA–PK activation

The exploration of physical interaction between EGFR and DNA–PK [34], which is a major enzyme of nonhomologous end-joining DNA–double-strand break repair, initiated the extended studies to understand the roles of nuclear EGFR in DNA repair and resistance to DNA-damaging radiotherapy and alkylators (Fig. 1). After treatment with cisplatin and irradiation, the interaction of EGFR with the catalytic subunit of DNA–PK (DNA–PKcs) and its regulatory heterodimeric complex Ku70/80 was observed in the nucleus in vivo and in vitro [34]. Because the EGFR NLS mutation interrupts the association of EGFR with DNA–PKcs and reduces the nuclear localization of DNA–PKcs, EGFR has been suggested to cotranslocate with DNA–PKcs into the nucleus and regulate the formation and activation of the DNA–PK complex after cisplatin treatment and IR [36]. Indeed, nuclear EGFR is associated with phosphorylation of DNA–PK at residue T2609, an indicator of DNA–PK activity during nonhomologous end-joining DNA repair [14,22]. Nuclear EGFR, in association with DNA–PK or Ku70/80, retains its intrinsic kinase activity [34]. Blocking of EGFR activation by its antibody cetuximab resulted in the decrease of DNA–PK activity, the increase of residual DNA damage, and the subsequent enhancement of the radiosensitivity of human A549 lung carcinoma cell line [22], suggesting that the tyrosine kinase activity of nuclear EGFR is required for the activation of the DNA–PK complex. However, the evidence revealing DNA–PK as a direct substrate of nuclear EGFR is lacking. In addition, EGFR tyrosine kinase activity may be essential but not sufficient for EGFR-dependent DNA–PKcs activation as overexpression of EGFR LNLS mutant, which contains both a constitutive activating mutation at L858 and an NLS mutation, cannot activate DNA–PKcs activity [36], further bolstering the crucial role of nuclear existence of EGFR in contributing to radioresistance and chemoresistance.

Although EGFR nuclear localization has been demonstrated to be required for modulation of cisplatin and IR-induced repair of DNA damage, the interaction between EGFR and DNA–PKcs was induced by cisplatin or IR but not by EGF stimulation or EGFR nuclear translocation per se [36]. Other mechanisms specifically elicited by DNA damage may be involved in the regulation of nuclear EGFR binding with DNA–PKcs. Interestingly, treatment with celecoxib, a COX-2 specific inhibitor, has been shown to obviously increase the radiosensitivity of multiple cancer cell lines via attenuating the radiation-induced EGFR nuclear transport and DNA–PK activation [72]. However, the radiosensitization by celecoxib seems to be independent of COX-2 activity [72]. Because celecoxib and its analogs possess an off-target effect on disrupting Akt signaling [73], which has been demonstrated to regulate EGFR nuclear import [21], it is worthy to further pursue whether radiation induces the interaction between nuclear EGFR and DNA–PK in an Akt-dependent manner.
5.2. **Nuclear EGFR functions as a transcription regulator to increase expression of gefitinib efflux pump**

Despite that the effects of nuclear EGFR on the sensitivity to gefitinib are not well understood, nuclear presence of EGFR seems to be a general event in different gefitinib-treated cells [21]. As illustrated in Fig. 1, we have reported that nuclear EGFR functions as a transcription regulator to mediate BCRP/ABCG2 [21] and thereby confers gefitinib resistance [44]. The expression of BCRP/ABCG2, a well-known transporter of ATP-binding cassette (ABC) family involved in chemoresistance to doxorubicin as well as many other chemotherapies [74,75], was found in 46% of advanced NSCLC patients [76]. Several studies have demonstrated that gefitinib is also a substrate of BCRP/ABCG2 [44–46] and can be pumped out of the cells, resulting in development of gefitinib resistance [44]. Aberrant expression of this transporter was not only correlated with the intrinsic insensitivity of wt EGFR-expressing patients to gefitinib [44] but also increased in the wt EGFR-expressing NSCLC patient with acquired gefitinib resistance [77–79], revealing BCRP as a valuable marker to predict the clinical outcome of gefitinib-treated patients without EGFR activating mutations and as a potential target to overcome the acquired resistance to gefitinib [44]. The BCRP promoter contains ATRSS and has been found to be targeted by nuclear EGFR in an Akt-dependent manner [21]. Mutation of EGFR NLS or silence of importin, which mediates the nuclear EGFR translocation, can abolish EGFR-dependent BCRP expression [21]. Although the promoter region of multiple drug resistance 1 (MDR-1/ABCB1), another ABC transporter, also contains ATRSS putative EGFR binding sites, the increase in MDR1 expression was not detected in the gefitinib-resistant cells [21]. Nuclear EGFR has been suggested to form a heteromeric transcription complex with the signal transducer and activator of transcription (Stat) proteins to mediate c-Myc expression in pancreatic cancer cells [80]. The overlapped binding site on BCRP promoter for Stat5 and ATRSS might account for the specific regulation of BCRP but not MDR-1 expression by nuclear EGFR [21].

6. **Perspectives and future directions**

The studies in nuclear functions of EGFR conducted in the past decade have disclosed several important pathological roles of the nuclear EGFR pathway in tumorigenesis. Since Dittmann and colleagues reported the involvement of nuclear EGFR in the activation of the DNA–PK complex to mediate the DNA repair in response to irradiation [14], a novel aspect of nuclear EGFR in the development of acquired resistance to anticancer therapies has emerged. This finding has hence evoked many other studies on the mode of action of nuclear EGFR in conferring resistance to chemotherapy and EGFR target therapy. These studies have had profound implications for the development of a novel strategy to overcome drug resistance by targeting nuclear EGFR signaling. Although researchers gain new insights into the therapeutic impact of nuclear EGFR on human cancers, many questions remain to be addressed:

1. Are there additional mechanisms underlying nuclear EGFR-mediated resistance? Although the regulations of DNA–PK activation and BCRP expression by nuclear EGFR have been demonstrated to mediate the resistance to DNA-damaging treatment and gefitinib, respectively, the detail mechanisms remain unclear. Novel nuclear proteins phosphorylated and functionally modulated by nuclear EGFR in response to anticancer treatments await to be discovered. In addition, several target genes or proteins of nuclear EGFR implicated in tumorigenesis have been identified. It is worthy to further pursue whether these known targets of nuclear EGFR also contribute to the formation of drug resistance.

2. Are there common mechanistic regulations that drive EGFR nuclear transport in response to different anticancer therapies? As described above, Akt activation has been viewed as a common signaling pathway to mediate the acquired resistance to multiple drugs and also plays a role in regulating gefitinib-induced EGFR nuclear translocation. Therefore, regulation by Akt may be a common mechanism for the EGFR import into the nucleus. However, other regulations, such as phosphorylation by SFK and PKCε, may compensate for this process.

3. Does nuclear EGFR function as a key regulator in cross-resistance among irradiation, chemotherapy, and EGFR target therapy? The nuclear EGFR-mediated BCRP expression in gefitinib-resistance cells has been found to cause the cross-resistance to doxorubicin. It would be of great interest to examine whether chemoresistant cells also exhibit nuclear EGFR and BCRP expression to reduce their sensitivity to gefitinib. In addition, the nuclear import of EGFR and the subsequent DNA–PK activation have been commonly observed in response to various treatments including irradiation and cisplatin. Further studies are needed to demonstrate whether the nuclear EGFR-mediated DNA–PK activation can result in the cross-resistance between these DNA-damaging therapies. Addressing this question will provide very important information to determine the use and priority of anticancer therapies.

4. Is there any efficient strategy for targeting nuclear EGFR signaling and thereby overcoming drug resistance? Once the nuclear role of EGFR in developing the resistance to anticancer therapies is extensively understood, blockage of nuclear EGFR signaling may be a new strategy to fight treatment resistance [23]. Indeed, targeting nuclear EGFR-dependent tyrosine phosphorylation of PCNA by blocking peptides has been shown to inhibit prostate cancer growth [81]. This finding revealed a promising strategy to overcome the nuclear EGFR-dependent resistance.

5. Do other plasma membrane receptors also function in the nucleus to confer the resistance to cancer therapies? In addition to EGFR, nuclear translocations of other plasma membrane-bound receptors, such as HER2 [17,55], ErbB4 [82–86], and fibroblast growth factor receptor (FGFR) [87], have also been observed in various cancer types and are associated with etiology and tumor progression of these cancers. It would be of interest to understand whether these receptors in the nucleus also play a crucial role...
in determining the cellular sensitivity to anticancer therapies.

Collectively, elucidation of these aspects of nuclear EGFR will help us evaluate the possibility of using nuclear EGFR as a biomarker to predict the sensitivity to various anticancer treatments and develop novel strategies to prevent or overcome the acquired resistance. If this is the case, nuclear EGFR may serve as a biomarker to help us stratify patients for personalized cancer therapy.

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References


Activation of Toll-like receptors induces macrophage migration via the iNOS/Src/FAK pathway

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Abstract

Macrophage motility is crucial in innate immunity. Toll-like receptors (TLRs), members of the interleukin-1 receptor superfamily, are a family of transmembrane pattern recognition receptors that are expressed primarily in antigen-expressing cells such as macrophages. These interleukin-1 receptors play a key role in immune response by recognizing highly conserved microbial molecules known as pathogen-associated molecular patterns. Studies have shown that when the macrophage-like cell line RAW264.7, peritoneal macrophages, and bone marrow-derived macrophages are exposed to lipopolysaccharide (LPS, TLR4 ligand), peptidoglycan (PGN, TLR2 ligand), polyinosinic–polycytidylic acid (polyI:C, TLR3 ligand), or CpG–oligodeoxynucleotides (CpG, TLR9 ligand), there is a marked increase in cell motility and in levels of activated Src (but not Fgr, Hck, and Lyn). Attenuation of Src suppressed LPS-, PGN-, polyI:C-, and CpG-elicited movement as well as the level of FAK Pi-Tyr861, which can be reversed by reintroduction of siRNA-resistant Src. Furthermore, siRNA knockdown of FAK decreased the degree of TLR-mediated motility of macrophages. Strikingly, LPS-, PGN-, polyI:C-, and CpG-enhanced Src expression, FAK Pi-Tyr861, and cell motility were greatly reduced in macrophages devoid of inducible nitric oxide synthase (iNOS, a NF-κB target), which can be induced by the aforementioned TLR ligands. Because NO can upregulate the expression and activity of Src, and because Src can mediate NF-κB activation, we hypothesize that there is a loop of signal amplification that influences the iNOS/Src/FAK axis for macrophage locomotion in response to engagement of TLRs.

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1. Introduction

The proto-oncogene product, c-Src, is a nonreceptor tyrosine kinase that is encoded by the cellular homolog (v-src) of the transforming gene of Rous sarcoma virus. Mounting evidence indicates that c-Src functions as a co-transducer of transmembrane signals emanating from a spectrum of polypeptide growth factor receptors, including the epidermal growth factor
neurons and platelets are known to retain high levels and activity of Src [2]. However, it has been shown that targeted disruption of the src gene in transgenic mice does not result in any obvious phenotypic or functional abnormalities except for osteopetrosis [4]. Osteopetrosis is a skeletal abnormality caused by a defect in osteoclasts [5], which are derived from hematopoietic precursors of the monocyte/macrophage lineage [6]. Osteoclasts function as resorbers of mineralized bone during bone homeostasis [7]. Deletion of Src leads to impaired bone resorption, which can be partly attributable to reduced osteoclast motility [7]. Although Src is indispensable in osteoclast function, its low basal expression conceals its physiological significance in macrophages. Recently, we demonstrated that nitric oxide synthase (iNOS)-induced activation of the Src and FAK pathway is responsible for macrophage migration in cells exposed to lipopolysaccharide (LPS). Strikingly, similar results were obtained in macrophages that had been exposed to peptidoglycan (PGN), polynosinic–polycytidylic acid (polyI:C), and CpG–oligodeoxynucleotides (CpG). In this review, we focus on the role that Src, FAK, and the upstream signaling protein iNOS play in macrophage motility in response to various Toll-like receptor (TLR) ligands.

2. Macrophages

Macrophages are important participants in innate immunity. They are ubiquitous and present in most tissues, and function as sensors of endogenous or exogenous danger signals by eliciting numerous immunological, inflammatory, and metabolic processes. In the developing embryo, the progenitors of macrophages differentiate in the yolk sac into monocytic tissue macrophages under the influence of granulocyte macrophage colony stimulating factor and macrophage colony-stimulating factor. In adults, pluripotent stem cells in bone marrow can develop into promonocytes (macrophage progenitors). Unlike the nonproliferating and short-lived monocyte-derived macrophages in inflammatory sites, tissue-derived macrophages maintain their numbers through homeostatic proliferation and appear to survive for at least 6 weeks [8]. With the ability to eliminate opsonized pathogens through diverse surface receptors and antigen presentation, macrophage recruitment to sites of infection as well as mobilization of activated macrophages from the infection area to the regional lymph node are crucial physiological processes in host defense. Disturbed regulation of this process results in pathological disorders such as atherosclerosis, sepsis, and autoimmune disorders.

3. Toll-like receptors

As crucial players in innate immunity, macrophages recognize invaded microorganisms via the interaction between the conserved structure called pathogen-associated molecular patterns (PAMPs) in microorganisms and their pattern-recognition receptors, such as TLRs [9]. TLRs contain N-terminal extracellular leucine-rich repeats that recognize specific pathogen components, a membrane-spanning domain that determines the cellular localization, and a C-terminal intracellular region similar to that of the interleukin-1 (IL-1) receptor known as the Toll/IL-1 receptor (TIR) domain, which is pivotal for downstream signaling [10]. A total of 12 TLRs have been identified in mice and a total of 10 have been identified in humans [9]. Certain TLRs (i.e., TLR1, 2, 4, 5, 6, and 11) are detected on the cell surface, whereas others (i.e., TLR3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as endosomes [9,11]. TLR2 can recognize PGN, a major bacterial cell wall component. TLR4 acts as a signaling receptor for LPS, an outer membrane component of Gram-negative bacteria [12,13]. TLR3 can sense viral double-stranded RNA [14] and TLR9 can detect the unmethylated CpG that are frequently found in bacteria, but not in vertebrate DNA [15]. Subsequent to recognition of a PAMP, TLR will recruit a combination of TIR-containing adaptors, including MyD88, TRIF, TRAM, and TIRAP/Mal. It is well-established that TLR2 utilizes MyD88, TLR3 needs TRIF, TLR2 requires MyD88 and TIRAP, and that TLR4 uses all four of the aforementioned adaptors [10]. Through individually preferential adaptors, engagement of TLRs triggers downstream signaling pathways that activate MAP kinase or NF-κB, which in turn produces proinflammatory cytokines required for host defensive strategies [9,11,16].

4. Inducible nitric oxide synthase

Nitric oxide (NO) is a unique diffusible messenger molecule that is produced via the oxidation of l-arginine by enzymes known as nitric oxide synthases (NOSs) [17]. Three distinct isoforms of the enzyme have been identified and characterized. Whereas Ca\(^{2+}\)/calmodulin can regulate the activity of constitutively expressed neuronal (n) and endothelial (e) NOS, the activity of inducible (i) NOS is independent of Ca\(^{2+}\)/calmodulin and is only induced by bacterial products as well as inflammatory cytokines. A low level of constitutively produced NO is a crucial mediator for a variety of physiological functions including regulation of neurotransmission, vasodilation, smooth muscle relaxation, and inhibition of platelet aggregation. In contrast, activated TLR pathways trigger a high level of NO production by transcriptional and post-transcriptional mechanisms that enhance the expression of iNOS in macrophages and other effector cells [18]. It is also well-established that some pathological processes such as inflammation and tumor development can be induced by sustained, chronically produced NO. Soluble guanylyl cyclase (sGC) is a cytosolic, heme-containing heterodimer comprising \(\alpha\) and \(\beta\) subunits whose activity can be modulated by NO. Binding of NO to the sGC heme prosthetic group activates sGC, which in turn leads to the conversion of guanosine 5′-triphosphate to the secondary intracellular messenger cGMP. Accumulation of cGMP results in transmission of NO signals to downstream effectors such as cGMP-regulated phosphodiesterase, cGMP-dependent protein kinase, and cGMP-gated cation channels, leading to physiological and pathological responses [19].

5. The Src family kinases

Cellular Src is a 60-kDa nonreceptor, membrane-associated tyrosine kinase that serves as the archetype for a group of Src
family tyrosine kinases (SFKs), including Yes, Fyn, Lck, Fgr, Hck, Lyn, and Blk [20]. Several SFKs are expressed in multiple isoforms because of the use of alternative start codons or alternative splicing patterns. Alignment of the amino acid sequence of the SFK members reveals their structural similarity. The conserved regions comprise an N-terminal myristylation signal that is responsible for the association of SFK with the plasma membrane; a unique region where the greatest sequence divergence among SFK members occurs; an SH3 domain and an SH2 domain that are responsible for direct protein–protein interaction; an SH2/kinase linker; an enzymatic domain; and a C-terminal regulatory region. Tyr416 and Tyr527 are two important phosphorylation sites that are located within the kinase domain and the C-terminal regulatory region, respectively. Unlike the self-mediated phosphorylation of Tyr416 (Pi-Tyr416) that enhances the catalytic activity of Src, CSK (a C-terminal Src kinase)-mediated phosphorylation of Tyr527 (Pi-Tyr527) downregulates Src activity. Based on mutational studies and X-ray crystallographic analyses, a model in which Src is held in an inactive conformation by the intramolecular association between SH2 and Pi-Tyr527 as well as SH3 and the SH2/kinase linker is proposed [21]. Disruption of these interactions will cause Src to become active and mediate Pi-Tyr416.

6. **Constitutive expression of myeloid-specific Src family kinases**

Src, Yes, and Fyn are widely expressed in most tissues, whereas the distribution of other members of the SFK family exhibits a more tissue-specific distribution, mainly in cells of hematopoietic lineage [22]. Lyn, Fgr, and Hck are myeloid-specific SFK members that are predominantly expressed in macrophages. Given that the release of eicosanoid mediators from LPS-treated RAW264.7 macrophages and the development of tumoricidal activity of LPS-stimulated murine peritoneal macrophages (PEMs) were impaired by herbimycin A (a tyrosine kinase inhibitor) [23] and that TLR4 did not possess intrinsic tyrosine kinase activity, Lyn, Fgr, and Hck seemed to be responsible for LPS-mediated tyrosyl phosphorylation and macrophage activation. Surprisingly, macrophages derived from mice with the combined deficiency of Lyn, Fgr, and Hck still retain full LPS responsiveness [24], a finding that implies that these three myeloid SFKs are not obligatory for LPS-evoked macrophage activation and that their exerted effects might be compensated by other tyrosine kinases.

7. **Inducible expression of Src**

Mounting evidence indicates that sustained activity of SFKs is indispensable for LPS-elicited responses [25]. Because knockout of Lyn, Fgr, and Hck does not hamper macrophage activation, nonmyeloid-specific SFKs are likely to compensate for the loss of their myeloid-predominant relatives. To circumvent the obstacle of their low basal protein expression as well as the need for their sustained activation, the potential tactic that macrophages might utilize is to have the nonmyeloid SFK members become LPS-inducible. Given that Src is indispensable for resorbing activity of macrophage-related osteoclasts, Src is likely to be the elusive and long-sought after SFK responsible for LPS-mediated macrophage activation. Indeed, LPS augments the expression of Src in both RAW264.7 macrophages and PEMs in a time-dependent manner. The upregulation of Src observed in macrophages recovered from LPS-challenged rats further implicates its physiological significance [25]. In agreement with the results obtained from lyn+/−/fgr+/−/hck−/− macrophages, Src with its expression induced by LPS, is an attractive, compensating candidate for the defect of Lyn, Fgr, and Hck. Intriguingly, Src induction is also detected in RAW264.7 macrophages, PEMs and bone marrow-derived macrophages (BMDMs) treated with PGN, poly(I·C), and CpG. This LPS-, PGN-, poly(I·C), and CpG-induced Src expression can be attributed to the increased level of the src transcript [26]. In contrast, the expression of Lyn, Fgr, and Hck is almost unaltered in RAW264.7, PEMs, and BMDMs in response to various TLR ligands [26]. The inducible characteristic of Src implicates its importance in relaying signals triggered by diverse PAMPs.

8. **Focal adhesion kinase**

Focal adhesion kinase (FAK) was initially identified as an Src target by immunological screening of the cDNA library with a panel of monoclonal antibodies directed against putative v-Src substrates [27]. FAK is an intracellular tyrosine kinase that participates in a wide-ranging spectrum of integrin-elicited biological activities including proliferation, survival, cell adhesion, and migration [28]. Deficiency of FAK results in embryonic lethality, characterized by impaired fibroblast motility [29]. Upon integrin stimulation, FAK becomes activated and autophosphorylated at Tyr397, the binding site for Src. This process facilitates Src-mediated FAK phosphorylation on several tyrosine residues including Tyr861, whose phosphorylation promotes FAK Pi-Tyr397 [30]. FAK was initially thought to be absent or expressed at low levels in monocyte/macrophages [31]. However, studies have shown that FAK is present in macrophages [32]. To study the physiological significance of FAK in macrophages, Owen et al. [33] generated myeloid-specific conditional FAK-knockout mice. Intriguingly, macrophages devoid of FAK displayed motility defects that coincided with reduced levels of adhesion turnover, elevated protrusive activity at the cell periphery, and a marked inability to form lamellipodia. In addition, recruitment of macrophages to sites of inflammation was shown to be impaired in the absence of FAK [33]. These findings indicate that FAK plays a major role in the regulation of macrophage mobility.

9. **The role of iNOS/Src/FAK axis in Toll-like receptor-mediated macrophage motility**

Based on the findings from seminal studies that NOS inhibitors suppress human monocyte chemotaxis [34] and that decreased polymorphonuclear granulocyte extravasation in peritoneal cavity was detected in zymosan-challenged iNOS-deficient mice [35], NO is now believed to be a key regulator of leukocyte locomotion during inflammation and tissue injury. Indeed, consistent with reduced LPS-, PGN-, poly(I·C), and CpG-
mediated macrophage migration by 1400 W (an iNOS-selective inhibitor), suppressed migration evoked by these TLR agonists was observed in iNOS null PEMs [26,36]. This iNOS deficiency caused defects in LPS-, PGN-, polyI:C-, and CpG-induced motility was not due to the general inability of cells to mobilize, because analogs of iNOS downstream mediators, SNAP (a NO donor) and 8-bromo-cGMP (8-Br-cGMP, a permeable cGMP analogue), were able to augment the migratory ability of iNOS-deficient macrophages in a way comparable to that of wild-type macrophages. These results indicated the participation of the NO/cGMP pathway in macrophage movement in response to activated TLRs [26,36]. Given that LPS-, PGN-, polyI:C-, and CpG-evoked macrophage motility was inhibited by PP2 (an SFK inhibitor), one interesting question turned out to be the relation between iNOS and SFKs. Because (1) LPS-, PGN-, polyI:C-, and CpG- induced Src induction was labile to 1400 W and ODQ (an sGC inhibitor); (2) Src, but not its myeloid relatives, could be induced by SNAP and 8-Br-cGMP; (3) there was a concomitant decrement of Src and cell migration in iNOS-null PEMs exposed to LPS, PGN, polyI:C, and CpG; and (4) the motility and Src induction in LPS-stimulated PEMs devoid of iNOS could be restored by SNAP and 8-Br-cGMP to a level comparable to that detected in wild-type PEMs inspired us to question whether Src might be the main SFK required for TLR-mediated NO/cGMP-dependent macrophage locomotion. Indeed, Src attenuation led to reduced LPS-, PGN-, polyI:C-, CpG-, and SNAP-evoked motility in RAW264.7 macrophages, and ectopically expressed avian Src rescued this defect. These results support the hypothesis that Src induction mediated by NO/cGMP is involved in macrophage mobilization in response to engagement of TLRs. As a downstream target of Src, the elevated level of Tyr861 phosphorylated FAK in LPS-, PGN-, polyI:C-, and CpG-treated macrophages was suppressed by Src attenuation and reversed by reintroduction of siRNA-resistant Src. The reduction of LPS-elicited FAK Pi-Y861 in both RAW264.7 and rat PEMs pretreated with iNOS inhibitor indicated that Src-mediated FAK Pi-Y861 might be responsible for LPS/iNOS-induced cell migration. Indeed, LPS-mediated FAK Pi-Y861 was inhibited in PEMs devoid of iNOS. Similar results were obtained in iNOS-null PEMs that had been stimulated with PGN, polyI:C, or CpG. Notably, FAK attenuation impaired macrophage motility in response to LPS, PGN, polyI:C, and CpG, revealing the importance of the iNOS/Src/FAK axis in TLR ligand-elicited macrophage migration.

**Fig. 1** — Engagement of TLRs leads to macrophage migration. As various PAMPs (i.e., LPS, PGN, polyI:C, and CpG) activate their respective TLRs, NF-κB will be activated and increase the expression of iNOS. NO, the second messenger generated by iNOS, will elevate the expression and activity of Src, which causes FAK activation and macrophage motility. Because Src increases the activity of NF-κB, thereby a signal amplification loop can be constructed. This model reveals a general mechanism utilized in macrophages when their TLRs are occupied.

### 10. Conclusions and future perspectives

In contrast to the large repertoire of rearranged receptors utilized by B and T cells in adaptive immunity, the innate immune response mechanisms recognize microorganisms via a limited number of germline-encoded PAMP recognition receptors including TLRs. Irrespective of their localization and utilization of different TIR-containing adaptors, engagement of TLRs lead to the activation of NF-κB and increased expression of iNOS, and proinflammatory cytokines. Given that the aforementioned TLRs are located on either plasma membranes (i.e., TLR2 and TLR4) or endosome (i.e., TLR3 and TLR9), and their mediated signaling pathways can be divided into MyD88-dependent (i.e., TLR2, TLR4, and TLR9) and -independent (i.e., TLR3) pathways, the iNOS-mediated upregulation of Src and activation of FAK in response to engagement of various TLRs might be a general mechanism of macrophage motility. Given that Src elevated the activity of NF-κB [37,38], thereby a signal amplification loop can be established. With what we have learned from studies conducted in murine RAW264.7 macrophage cell line as well as PEMs recovered from normal versus iNOS−/− mice, a simple model illustrating the responsible mechanism for TLR ligands-ignited, iNOS-dependent macrophage motility was proposed in Fig. 1. Considered mobilization of activated macrophages from infection sites to the regional lymph node where they present antigens to naïve CD4+ T cells is an essential and crucial step in activating the adaptive immunity [11]; therefore, we speculate that the iNOS → Src → FAK signaling pathway might be an important general mechanism utilized by TLR-engaged macrophages required in host defenses. Considering the fact that the accumulation of macrophages contributes to progression of diseases such as cancer and chronic inflammatory diseases, our data highlight the potential
of iNOS, Src, and FAK as therapeutic targets to control infection, inflammation, and the maintenance of tissue homeostasis.

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References

P2X7 receptor as a double-edged sword: Neurotrophic and neurotoxic effects

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Strong activation of the P2X7 receptor (P2X7R) by extracellular ATP is excitotoxic to neurons during brain ischemia or in spinal cord injury. In addition, activation of P2X7R in microglia is known to cause neuroinflammation and consequently neurodegeneration. This review focuses mainly on the direct effects of activation of P2X7R in neurons. P2X7R is an ATP-gated, nonselective ion channel that provides an influx pathway for Na$^{+}$ and Ca$^{2+}$, and upon sustained ATP stimulation, dilates into a pore permeable to molecules up to 900 Da. Mechanisms governing P2X7R-mediated cell death due to Ca$^{2+}$ overload in the cytosol and possible involvement of perturbation of Ca$^{2+}$ homeostasis in the endoplasmic reticulum and mitochondria are also discussed. In contrast to strong P2X7R activation, basal and mild activation of the P2X7R have been known to exert both antiapoptotic and proliferative effects in cancer cells, glial cells, and some neuroblastomas. How these effects are related to endoplasmic reticulum and mitochondrial Ca$^{2+}$ homeostasis are described. In addition, the putative role that mild P2X7R activation plays as a neurotrophic signal is discussed.

1. Introduction

Extracellular ATP not only functions as a neuromodulator or cotransmitter in cholinergic, gamma-aminobutyric acid (GABA)-ergic, adrenergic, and glutamatergic nerve terminals, but also acts as a fast neurotransmitter in its own right in the central as well as the peripheral nervous system [1–3]. The storage of ATP in synaptic vesicles, Ca$^{2+}$-triggered release, extracellular degradation by ecto-ATPases, and versatile signaling through pre- or postjunctional membrane receptors all indicate that this simple molecule is a neurotransmitter [3,4].

In addition to its physiological roles, a number of pathophysiological effects have been attributed to ATP signaling (for reviews, see [3,4]). Among these, ATP has been shown to be involved in excitotoxicity in neural tissues. In brain ischemia and in stroke, excitotoxicity occurs when a hyperactivation of glutamate receptors leads to cytosolic Ca$^{2+}$ overload and neuronal cell death. What complicates the situation is that during brain ischemia/hypoxia, extracellular ATP levels also increase [5]. Pathologically high levels of ATP can lead to or worsen excitotoxicity [5,6]. Extracellular ATP activates neuronal and glial cell ATP receptors, such as metabotropic P2Y and ionotropic P2X receptors. P2X receptors have been known to play important physiological roles in neurotransmission, and are classified into P2X1–P2X7 [7]. Excessive amounts of extracellular ATP in pathological situations cause strong and chronic activation of certain P2X receptors, eventually leading to neurotoxicity.

The P2X7 receptor (P2X7R) in particular has attracted recent attention. Activation of P2X7R, a cation channel, elicits...
substantial Ca\textsuperscript{2+} influx and exacerbates excitotoxicity. The P2X7R has traditionally been regarded as a cytotoxic receptor or a “cell death receptor.” For instance, P2X7R stimulation in vivo is lethal to rat retinal ganglion neurons, especially in hypoxic conditions [8,9]. Activation of the P2X7R, together with nerve growth factor, aggravates hypoglycemia-induced cell death in cerebellar granule neurons [10]. P2X7R stimulation also causes necrotic death in SN4741 dopaminergic neurons derived from the substantia nigra of transgenic mouse embryos [11] and accounts for neurotoxicity in spinal cord injury [12,13]. P2X7R stimulation is also cytotoxic to neural progenitor cells, thereby preventing successful implantation [14]. Interestingly, it has been shown that activation of P2X7R induces death of retinal cholinergic neurons during normal development, thus regulating the optimal density of these neurons [15]. P2X7R stimulation is detrimental not only to neurons, but also to glial cells. For instance, P2X7R stimulation is cytotoxic to microglial cells [16] and causes damage to oligodendrocytes [17,18]. What worsens the situation is that, during cerebral ischemia, P2X7R expression has been shown to be upregulated [19]. Furthermore, in vitro ischemia has also been shown to enhance the sensitivity of P2X7R to ATP [20].

It is conceivable that inhibition of P2X7R stimulation would offer neuroprotection. Thus, downregulation of P2X7R expression by retinoic acid might suppress cell death induced by 2',3'-O-(4-benzoylbenezoyl)-ATP (BzATP, a selective P2X7R agonist) in human SH-SYSY neuroblastoma cells [21]. Substantial ATP release and the subsequent activation of P2X7R have been shown to account in part for the neurotoxicity after spinal cord injury, and there is evidence that significant improvement in spinal cord function can be achieved following pharmacological blockade of P2X7R [12,13].

2. The mechanism of P2X7R-mediated cytotoxicity

P2X7 receptors are distributed throughout the nervous system. These receptors are present in glial cells (astrocytes, microglia, and Schwann cells) as well as in sensory neurons, neurons in the brainstem, spinal cord, cerebral cortex, hippocampus, amygdala, cerebellum, thalamus, and several neuroblastoma cell lines [22]. One of the causes of P2X7R-mediated neurotoxicity is via activation of these receptors in microglial cells, which causes neuroinflammation (e.g., release of interleukin-1) and subsequent neurodegeneration. Therefore, pharmacological targeting of the P2X7R signaling pathway offers potential therapeutic opportunities for treating neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease (for recent reviews, see [23,24]). This mechanism is not discussed in the present review article, but readers can consult recent reviews on this topic [23,24]. The other cause of P2X7R-mediated lethality is the direct death signal to the glia or neurons expressing P2X7R upon activation [22]. This review focuses mainly on the direct effect of P2X7R activation on neurons.

The P2X7 receptor is a nonselective cation channel pore that allows the influx of Na\textsuperscript{+} and Ca\textsuperscript{2+}. It is a homotrimer that becomes activated upon the binding of three ATP molecules [25]. Each subunit has two transmembrane domains (TM1 and TM2), intracellular C- and N-termini and a large chunk of extracellular domain; the latter from each of the three subunits intertwine to surround a vertical cavity at the center.

P2X7R opens as a cation channel in response to high micromolar levels of ATP [25]. Opening of the channel allows for the influx of Ca\textsuperscript{2+} and Na\textsuperscript{+}, and the resulting depolarization causes the opening of voltage-gated Ca\textsuperscript{2+} channels, which eventually leads to Ca\textsuperscript{2+} overload inside the cell. The high intracellular concentration of Ca\textsuperscript{2+} causes activation of nucleases and proteases (e.g., calpains), which results in cell death [26]. Ca\textsuperscript{2+} overload also leads to mitochondrial uptake of Ca\textsuperscript{2+} and the collapse of mitochondrial membrane potential, which eventually culminates in caspase activation and apoptosis [27]. Upon sustained exposure to millimolar concentrations of ATP, the P2X7R, instead of being desensitized, further “dilates” to a nonselective pore permeable to much larger ions such as N-methyl-D-glucamine; in fact, the dilated pore allows for the passage of hydrophilic molecules of up to 900 Da [7]. The mechanism for such pore dilation is not yet fully understood, but may involve rotation and separation of the transmembrane helices in the presence of continued ATP binding [25]. The consequent efflux of small and essential cellular constituents through the dilated pore inevitably aggravates cellular toxicity.

Another consequence of excitotoxicity is the perturbation of intracellular organelles that are responsible for handling Ca\textsuperscript{2+}, such as endoplasmic reticulum (ER) and mitochondria. Interestingly, a moderately augmented filling of the ER and mitochondria provides a pathway for P2X7R-triggered cell proliferation (see the section on basal or mild P2X7R stimulation as a neurotrophic signal).

3. Perturbation of ER and mitochondrial Ca\textsuperscript{2+} homeostasis

The ER performs several essential functions, namely, synthesis of proteins, post-translational modification and folding of proteins, degradation of malfolded proteins, and also intracellular Ca\textsuperscript{2+} storage. Many hormones or neurotransmitters, by activating G-protein-coupled receptors that are associated with phospholipase C, generate inositol-1,4,5-trisphosphate (IP3) and diacylglycerol from phosphatidyil-4,5-bisphosphate (PIP2) cleavage. IP3 binds to its receptor (IP3R, a Ca\textsuperscript{2+}-release channel) in the ER and mobilizes Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores, while diacylglycerol activates protein kinase C (see [28] for a review). This bifurcating signaling pathway is responsible for the physiological versatility of many neurotransmitters and hormones.

After Ca\textsuperscript{2+} mobilization from the store by IP3, the emptiness of the Ca\textsuperscript{2+} store triggers the opening of a store-operated Ca\textsuperscript{2+} channel at the plasma membrane; this channel is now believed to be composed of the proteins stromal interaction molecule (as a sensor of the Ca\textsuperscript{2+} content in the store) and Orai (the channel protein molecule at the plasma membrane; Orai is tethered to the stromal interaction molecule) [29]. Besides the IP3-sensitive Ca\textsuperscript{2+} pool, there is also the ryanodine-sensitive pool; millimolar levels of caffeine or low micromolar concentrations of ryanodine bind to the ryanodine
receptor (RyR, a \( \text{Ca}^{2+} \)-release channel), which results in the release of intracellular \( \text{Ca}^{2+} \). The physiological agonist of RyR has been proposed to be cADP-ribose (for a review, see [30]). Interestingly, cytosolic \( \text{Ca}^{2+} \) acts as a co-agonist of IP3R and RyR. Therefore, high cytosolic \( \text{Ca}^{2+} \) levels could result in a \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release (CICR) phenomenon [28,30].

Stress to the ER can be caused by hypoxia, toxins, protein synthesis overload, mutations causing protein malfolding, and perturbations of ER \( \text{Ca}^{2+} \) stores [21]. Sustained reduction in \( \text{Ca}^{2+} \) content inside the ER is a strong ER stress signal. For example, cyclosporin A, which acts as a \( \text{Ca}^{2+} \) store depletor by inhibiting the sarcoplasmic/ER \( \text{Ca}^{2+} \) ATPase, can induce ER stress by emptying the ER of its store of \( \text{Ca}^{2+} \). It should be noted that \( \text{Ca}^{2+} \) overload could potentially, via the CICR mechanism mentioned above, aggravate neuronal excitotoxicity by releasing more stored \( \text{Ca}^{2+} \) into the cytosol and by causing more depletion of the ER \( \text{Ca}^{2+} \) pool [32], presumably causing more ER stress.

Whether, and to what extent, ER stress contributes to excitotoxicity in neurons is not fully understood. For instance, whether glutamate-mediated neuronal excitotoxicity involves ER stress still remains controversial. Kainic acid, a glutamate receptor agonist, causes hippocampal neuronal cell death, disintegration of the ER membrane, and appearance of ER stress proteins such as Bip, C/EBP homologous protein, and caspase-12 [33]. N-methyl-D-aspartate (NMDA), an agonist of another glutamate receptor subtype, has been shown to induce ER stress in rat retinal ganglion RGC-5 cells [34]. Conversely, it has been demonstrated in vitro and in vivo that NMDA receptor-mediated excitotoxic apoptosis of neurons in rat cerebellum, cortex, and hippocampus occurs without any indication of ER stress [35]. ATP-mediated (through P2X7R) neuronal excitotoxicity has been recognized and is known as one of the multiple factors that cause excitotoxicity [5,17]. However, no published information is hitherto available about whether P2X7R-mediated neurotoxicity involves ER stress. We have found that exposure of differentiated neuronal NG108-15 cells to 100 \( \mu \text{M} \) BzATP leads to P2X7R activation and the subsequent depletion of \( \text{Ca}^{2+} \) stores, ER stress, and eventually cell death (unpublished observation). Further research is needed to investigate how \( \text{Ca}^{2+} \) stores are depleted and whether the depletion involves the CICR mechanism.

\( \text{Ca}^{2+} \) overload in neurons may also lead to an increase in mitochondrial \( \text{Ca}^{2+} \) levels via \( \text{Ca}^{2+} \) transfer from the ER to the mitochondria [36]. \( \text{Ca}^{2+} \) release via the IP3R creates a very high \( \text{Ca}^{2+} \) concentration in the ER mitochondria cleft, which allows \( \text{Ca}^{2+} \) to enter the mitochondria via a complex formed by the IP3R and voltage-dependent anion channel (VDAC, located in the outer mitochondrial membrane). \( \text{Ca}^{2+} \) subsequently enters the matrix, presumably via the \( \text{Ca}^{2+} \) uniporter in the inner mitochondrial membrane [36]. When the mitochondrial matrix \( \text{Ca}^{2+} \) level reaches a toxic threshold, the permeability transition pore opens and leads to loss of ion and solutes to the cytosol, collapse of mitochondrial membrane potential, and activation of the apoptotic cascade [36]. It would be interesting to evaluate whether strong P2X7R activation in neurons leads to a \( \text{Ca}^{2+} \) transfer from ER to mitochondria, and if that is the case, how it occurs. Does it occur via the IP3R–VDAC complex? Further investigations are needed to answer such questions.

### 4. Basal or mild P2X7R stimulation as a neurotrophic signal?

Although high (i.e., millimolar) levels of ATP have been shown to be cytotoxic, studies by Di Virgilio and colleagues in the past 10 years have revealed that the basal activities of the P2X7R cause antipoptotic as well as proliferative effects. For example, transfection of P2X7R-deficient lymphoid cells with P2X7R was shown to lead to cell proliferation in the absence of serum [37]. This initially surprising finding was subsequently confirmed by studies showing that several tumor cells had high levels of P2X7R expression [38–40]. The presence of P2X7R (by transfection of P2X7R in HEK293 cells) allows a tonic, albeit mild, stimulation resulting in raised membrane potential and \( \text{Ca}^{2+} \) levels in the mitochondria, and higher intracellular ATP content, and allows cells to grow in serum-free medium [39]. Such basal stimulation is maintained by the autocrine release of endogenous cellular ATP, since degradation of extracellular ATP by apyrase abolishes cell proliferation. Chelation of extracellular \( \text{Ca}^{2+} \) by EGTA also abolishes cell growth. It is, therefore, hypothesized that the tonic activation of P2X7R allows for a continuous small flux of \( \text{Ca}^{2+} \) into the mitochondria, which mildly raises the mitochondrial [\( \text{Ca}^{2+} \)], and leads to increased oxidative phosphorylation and increased mitochondrial potential, and thus increased levels of ATP production. The enhancement in energy production is beneficial for cell growth and survival.

However, if the P2X7R-transfected cells are strongly stimulated by exogenous ATP, cytosolic [\( \text{Ca}^{2+} \)] markedly increases and mitochondria become overloaded with \( \text{Ca}^{2+} \), leading to a collapse of mitochondrial membrane potential and eventually apoptosis [39]. Interestingly, in the same study, it was found that the P2X7R transfectants and the mock control did not differ in their basal cytosolic [\( \text{Ca}^{2+} \)], suggesting that the weak flux of extracellular \( \text{Ca}^{2+} \) through the P2X7R could be taken up into the mitochondria, possibly via the ER, without causing any elevation in cytosolic [\( \text{Ca}^{2+} \)]. The above-mentioned proposal that \( \text{Ca}^{2+} \) passes through the ER to reach the mitochondria is supported by further findings by the same group that P2X7R transfection in HEK293 cells augments \( \text{Ca}^{2+} \)-filling of the ER (i.e., resulting in higher ER \( \text{Ca}^{2+} \) levels) [41]. \( \text{Ca}^{2+} \) transfer from ER to mitochondria may occur via the IP3R–VDAC complex (see the previous section). P2X7R transfectants are more resistant to ceramide- and staurosporine-induced apoptosis; they also respond to agonists with larger \( \text{Ca}^{2+} \) signals in the cytosol and mitochondria, offering these transfectants survival advantages upon stimulation by \( \text{Ca}^{2+} \)-mobilizing agents [41]. Those authors also observed that, in the P2X7R transfectants, there was a stronger activation of nuclear translocation of nuclear factor of activated T cell complex 1 (NFATc1); this activation is considered essential for growth since pharmacological blockade of NFATc1 activation has been shown to abolish the growth effect in P2X7R transfectants.

Does stimulation of P2X7 receptors also enhance cell growth of or protect neuronal cells? Raffaghello et al. [42] have shown that P2X7R stimulation by millimolar levels of ATP or BzATP does not kill but causes \( \text{Ca}^{2+} \) elevation, plasma membrane depolarization, and enhanced growth of human
addition, it has been reported that overexpression of P2X7R has been shown to markedly inhibit microglial growth [44]. In P2X7R expression or pharmacological blockade of P2X7R has led to enhanced cell survival, suggesting a trophic effect. For example, small interfering RNA knockdown of P2X7R expression or pharmacological blockade of P2X7R has led to enhanced cell survival, suggesting a trophic effect.

More recently, P2X7R activation was also shown to be important in sustaining mouse N2a neuroblastoma cell viability [43]. We have been investigating whether weak stimulation of P2X7R could be neuroprotective in differentiated neuronal cell lines. In NG108-15 cells induced to differentiate by retinoic acid and forskolin, cells cease to divide and begin to develop long neurites characteristic of neurons. As shown in Fig. 1, differentiated control cells died over time when they were kept in low-serum (0.5% fetal bovine serum)-containing culture medium. Addition of a low concentration (5 μM) of BzATP increased cell survival significantly at day 4. This result suggests that weak P2X7R stimulation could be neuroprotective. We are in the process of delineating the protective mechanisms involved. Does the protection happen via mildly enhancing the filling of ER and mitochondria? It will also be of importance to examine whether similar protection would occur in primary neuron cultures. On the other hand, it would be interesting to examine whether a mild and tonic activation of P2X7R could provide a trophic signal to neural progenitor cells.

Basal P2X7R stimulation can also lead to microglial proliferation. For example, small interfering RNA knockdown of P2X7R expression or pharmacological blockade of P2X7R has been shown to markedly inhibit microglial growth [44]. In addition, it has been reported that overexpression of P2X7R alone (without inflammatory stimuli) suffices to stimulate proliferation of microglia in rat primary hippocampal cultures; this trophic effect has been shown to be abolished by selective P2X7R antagonists [45].

Fig. 1 – NG108-15 cell death due to incubation in low serum (0.5% fetal bovine serum) was partially reversed by a low concentration of 2',3'-O-(4-benzoylbenzoyl)-ATP (BzATP). Cells were incubated in medium containing only 0.5% FBS with or without 1 or 5 μM BzATP, and cell viability was determined by a trypan blue exclusion test on different days. Results are mean ± standard error from four separate experiments.

5. Conclusion and perspectives

The basal activities, or weak stimulation, of P2X7R appear to be important for the growth of a number of cancer cells, including neuroblastoma cells. Such mild activation may not involve large pore formation, and would allow a small Ca2+ influx to enhance the filling of ER and the subsequent raising of mitochondrial Ca2+ levels. Whether this mild stimulation of P2X7R is neuroprotective or even beneficial to the growth of neural progenitor cells warrants future in-depth exploration. Basal or weak P2X7R activities appear to promote glial cell activation and proliferation, but may lead to neuro-inflammation and eventually neurodegeneration. Strong stimulation of P2X7 receptors, which is likely to involve large and nonselective pore formation, leads to cell death of both neurons and certain glial cells. Therefore, inhibition of P2X7R stimulation would offer dual neuroprotection: prevention of neuronal cell death and suppression of microglial activation. If mild P2X7R stimulation is trophic to neural progenitor cells, caution has to be exercised when P2X7R antagonists are administered as potential neuroprotective drugs.

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References

[9] Sugiyama T, Ooku H, Shibata M, Fukuhara M, Yoshida H, Ikeda T. Involvement of P2X7 receptors in the hypoxia-
Mechanisms governing the protective effect of 17β-estradiol and estrogen receptors against cardiomyocyte injury

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\textbf{A B S T R A C T}

The sex hormone 17β-estradiol (E2) is the most abundant and active estrogen in premenopausal women. Studies have shown that high circulating levels of E2 are cardioprotective and are associated with reduced risk of developing heart disease in women of reproductive age. Estrogen receptors (ERs) are divided into three subtypes, namely ER\textsubscript{a}, ER\textsubscript{b}, and GPR30, and these receptors have been shown to play important roles in E2-mediated pathways that protect cardiomyocytes from various cardiac insults, such as hypoxia, ischemic-reperfusion injury, sepsis, and hypertrophic agents. This review focuses on the role that estrogen and ER-mediated signaling pathways play in protecting cardiomyocytes against various stresses.

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\textbf{1. Introduction}

Heart disease is a major cause of death worldwide and usually develops as a result of deteriorating myocardial function. The incidence of heart disease is low in premenopausal women but increases substantially in postmenopausal women, suggesting that sex steroid hormones protect the female heart [1]. Evidence from in vivo and in vitro studies suggests that 17β-estradiol (E2), the most abundant and active estrogen in premenopausal women, plays a cardioprotective role by...
preventing cardiomyocyte apoptosis and alleviating left ventricular hypertrophy, as well as protecting against the development of cardiac fibrosis in women. Nevertheless, a few clinical trials on the effect of estrogen replacement in postmenopausal women have shown that high levels of estrogen may contribute to the development of heart disease [2,3]. Further investigations are warranted to fully understand the complex effects of estrogen and estrogen receptors (ERs) on cardiomyocyte biology prior to the clinical application of estrogen for treatment of cardiomyopathy. This article will review the current state of knowledge of estrogen signaling in cardiomyocyte protection.

2. Molecular mechanisms of ER signaling

The physiological actions of E2 are mediated by two ER subtypes, ER-alpha and ER-beta (ERα and ERβ), and ER signaling can be divided into genomic and nongenomic pathways. In genomic ER signaling, estrogen diffuses into cells and binds to ERs to form a nuclear estrogen–ER complex. This complex then binds to estrogen response element (ERE) sequences in the regulatory regions of estrogen-responsive genes, with consequent physiological responses. Another type of ER genomic activity occurs through protein–protein interactions with activator protein 1 (AP1) or specificity protein 1 (SP1) sites in the promoter region of estrogen-responsive genes. Both ERα and ERβ can modulate gene expression either via ERE-mediated signaling or by interacting directly or indirectly with other transcription factors. This mechanism represents the tight regulation of ER signaling in response to 17β-estradiol.

In nongenomic ER signaling, estrogen binds to ERs via cytoplasmic signal transduction proteins, such as mitogen-activated protein kinase, Stats (signal transducers and activators of transcription), and Src family tyrosine kinases, or through membrane-associated estrogen-binding receptors, resulting in cellular responses [4]. Binding of ERs to the p85 subunit of type I phosphoinositide-3 kinase (PI3 K), which results in increased PI3 K activity and subsequent activation of protein kinase B (Akt), governs E2- and ER-mediated pro-survival signaling in cardiomyocytes [5]. In contrast, ER signaling can be activated by growth factors regardless of the presence of E2. Growth factors such as insulin-like growth factor 1 (IGF1) and epidermal growth factor (EGF) interact with their membrane-bound receptors (receptor tyrosine kinases) and induce mitogen-activated protein kinase signaling, which in turn activates ER by changing its phosphorylation status [6].

GPR30 (G protein-coupled estrogen receptor) is a membrane-localized ER located primarily on plasma membranes, endoplasmic reticulum, and nuclear membranes [7]. This membrane-bound ER has high E2 affinity but low ligand capacity, and is considered to play an important role in rapid signaling events and rapid transcriptional activation. The role that GPR30 plays in E2-mediated cardioprotection, however, is not clear.

3. Structure of ERs

ERα and ERβ are transcribed from different genes and display distinct expression patterns as well as different ligand specificities [8]. These two ER subtypes are members of the nuclear receptor superfamily and share common structural characteristics, including five distinguishable domains, which are defined as the A/B, C, D, E, and F domains, respectively [9]. The A/B domain is thought to contribute to ER subtype-specific actions on target genes, and the two ER subtypes share less than 20% amino acid homology in this region. The domain contains activation function 1 (AF1), which provides for ligand-independent ER activation [10]. The A/B domain is associated with the development of tamoxifen resistance in some breast cancer patients. Studies have shown that excessive activation of growth factors may give rise to the possibility of ligand-independent ERα activation via that ligand-independent pathway [11].

The central C domain is highly conserved among two of the ER subtypes and is critical for specific DNA binding and dimerization. The D domain is the hinge domain between the DNA-binding domain and the ligand-binding domain, and is considered to play an important role in ER nuclear translocation [12,13]. The E domain is referred to as the ligand-binding domain, and ERα and ERβ share approximately 55% amino acid identity in this region. The ligand-binding domain contains a hormone-dependent activation function 2 (AF2) domain that is important for ligand binding. The F domain has less than 20% amino acid identity between the two ER subtypes, and the functions of this domain remain unknown.

GPR30 was initially identified as an orphan G protein-coupled receptor with estrogen as its endogenous ligand. As a transmembrane ER, GPR30 activation may mediate E2 rapid cell signaling [14]. The effects of and the molecular mechanisms governing GPR30 in cardiomyocytes have not been fully evaluated.

4. E2/ER protects against lipopolysaccharide-induced cardiomyocyte death

Lipopolysaccharides (LPSs) are a common cause of sepsis and a common cause of sepsis-induced heart failure [15]. This outer-membrane component of Gram-negative bacteria is known to interact with toll-like receptor 4 (TLR4) on cardiomyocytes, resulting in inflammation and cardiomyocyte apoptosis [16]. Liu et al found that LPS-induced myocardial apoptosis was mediated by c-Jun N-terminal kinases 1/2. Their studies showed that JNK1/2 activated nuclear factor kappa B (NFκB), which in turn led to the release of cytochrome C as well as the overexpression and activation of pro-apoptotic proteins such as tumor necrosis factor alpha (TNFα), caspase 8, truncated BH3 interacting domain death agonis bid (t-Bid), Bcl2-associated X protein (BAX), caspase 9, and caspase 3 [17]. Elevated PI3 K-Akt activity mediated by E2 and ERs contributes to the inhibition of nuclear translocation of NFκB, and therefore diminishes LPS-induced apoptosis of cardiomyocytes (Fig. 1). This finding is consistent with that reported by Pelzer et al, who showed that E2 and ERs inhibit the nuclear localization of NFκB [18]. Their finding might explain why menopausal women with sepsis have lower mortality rates as well as a lower incidence of heart failure.
Interestingly, the ERα receptor and membrane-impermeable E2 seem to act synergistically through a similar signaling pathway to protect against LPS-induced damage in cardiomyocytes. On the other hand, previous studies have shown that selective activation of ERβ also inhibits nuclear translocation and DNA binding of NFκB in cardiomyocytes [19]. Further studies on whether ERβ can protect against LPS-induced heart disease are warranted.

5. **E2/ERs protect against hypertrophic stimuli-induced cardiac hypertrophy and cardiomyocyte death**

Cardiac hypertrophy is one of the most frequent causes of heart failure and can arise from a variety of cardiac insults including hypertension, excess activation of the sympathetic nervous system, or other hypertrophic agents such as angiotensin II, endothelin 1, or β-adrenergic receptor agonist [20–22]. Cardiac hypertrophy can be characterized as an alternation in cardiac geometry (size and shape) with increases in cardiomyocyte size as well as extracellular matrix components. Pathological hypertrophy often results in cardiomyocyte apoptosis and eventually to deterioration of cardiac function.

The importance of abnormal activity of calcineurin (a calcium-sensitive phosphatase; PP2B) in cardiac hypertrophy has been investigated intensively. Calcineurin activity is increased by a variety of hypertrophic stimuli, such as angiotensin II and isoproterenol [23]. Calcineurin activation promotes the nuclear translocation of nuclear factor of activated T cells 3 (NFAT3) and activates myocyte-enhancing factor 2, resulting in the upregulation of hypertrophic genes [23,24].

The results from various animal studies indicate that estrogen may defend against the development of cardiac hypertrophy. We recently showed that E2 and ERβ alleviate isoproterenol-induced cellular calcium accumulation in cardiomyocytes by activating phospholamban (PLB) and PI3 K-Akt-murine double minute 2 (MDM2) signaling cascades...
[25], which increase the protein degradation of calcineurin, thereby inhibiting isoproterenol-induced myocardial cell hypertrophy and apoptosis (Fig. 2). On the other hand, ERα seems to protect against isoproterenol-induced hypertrophy and apoptosis in myocardial cells. Our laboratory has recently discovered that E2 facilitates the interaction between ERα and Src proteins in myocardial cells, and that such interactions result in the activation of the IGFIR-Pi3 K-Akt and EGFR-MMP2/9-MEK1/2-ERK1/2 signaling pathways. The activated pathways mentioned above markedly decrease the levels of calcineurin-induced proapoptotic proteins and play a role in protecting cardiomyocytes from isoproterenol-induced apoptosis [26].

Interestingly, Filardo et al reported that GPR30-mediated G-protein βγ subunits (Gβγ) activation by E2 results in the activation of Src and matrix metalloproteinase-mediated cleavage of heparin-bound EGF. The latter is then able to activate the EGF receptor with subsequent acute activation of PI3 K and extracellular signal-regulated kinases (ERK) [27]. This pathway is very similar to what we have observed in E2- and bovine serum albumin (BSA)-E2-treated cardiomyocytes as well as in tetracycline-inducible gene expression system (Tet-on) cardiomyoblast cells that overexpress ERα, suggesting that the EGFR-MMP2/9-MEK1/2-ERK1/2 pathway might be the crucial pro-survival signal for E2 and ERs in cardiomyocytes in response to hypertrophic insults. These data raise an interesting question concerning the cardioprotective effects of GPR30 against hypertrophic insults.

In addition, we have also identified a novel ERα-mediated cardioprotection mechanism (Fig. 3). Under isoproterenol stimulation, activated ERα interacts with glycogen synthase kinase 3 beta (GSK3β) in cardiomyocytes, with subsequent upregulation of I2PP2A (a potent inhibitor of protein phosphatase [PP] 2A). This prevents PP1 activation by PP2A and contributes to the stabilization of intracellular calcium concentration by suppressing the association between PP2A and NCX (a cardiac Na⁺/Ca²⁺ exchanger), the consequent activation of isoproterenol-induced calcineurin, as well as apoptosis [28].

6. E2/ERs protect against myocardial oxidative stress and ischemic-reperfusion injury

Ischemic heart disease (IHD) is the most common cause of death and hospitalization in many Western countries [29]. Reduced blood supply induces a hypoxic situation in heart muscle, which usually stimulates the production of cytokines, such as interleukin 6, and subsequently leads to myocardial inflammation and cardiomyocyte apoptosis [30,31]. In addition, re-establishment of blood flow in infarcted myocardial tissue can paradoxically cause further damage to ischemic tissue, a condition referred to as 'ischemia-reperfusion injury'. Epidemiological studies indicate that premenopausal women are at a lower risk of developing IHD than men of similar age [32]. Furthermore, favorable effects of estrogen replacement therapy, including smaller infarct size and reduced apoptosis in the peri-infarct zone of the left ventricle, have been shown in a few animal models of ischemia-reperfusion injury [33]. These preclinical as well as epidemiological studies suggest that estrogen has a cardioprotective effect against myocardial ischemia-reperfusion injury.

Reactive oxygen species (ROS) are the primary cause of cardiomyocyte death during the reperfusion stage of ischemia-reperfusion injury [34,35]. Attenuation of ROS generation as well as the increased activity of the GSH/GRX (γ-glutamylcysteinylyglycine/glutaredoxin) system is thought to play an important role in E2- and ER-mediated cardioprotection against ischemia-reperfusion injury. Liu et al suggested that E2 activation of ERα or ERβ results in the activation of PI3 K with subsequent inhibition of ischemia-reperfusion injury-induced ROS [36]. This ER-mediated protection can be summarized as follows: First, activated PI3 K increases p38β activity and downregulates the activity of p38α, leading to inhibition of p53 activation with subsequent ischemia-reperfusion-induced cardiomyocyte apoptosis. Second, increased levels of S-nitrosylation proteins by activation of endothelial nitric oxide synthase (eNOS) results in decreased oxidative stress and ROS generation [37]. In addition, E2 also mediates cardioprotection either through ERα or ERβ genomic pathways. Activation of either ERα or ERβ upregulates the expression of gamma-glutamylcysteine synthetase (γ-GCS) and glutathione S-transferase (GSH) and then activates the GSH/GRX system, resulting in reduction of the oxidative state of Akt; this thereby inhibits PP2A activation and protects cardiomyocytes from oxidative stress-induced apoptosis by preserving Akt activity. Meanwhile, ERα interacts with AP1 and SP1 on γ-GCS and GSH genes, and ERβ binds to an EpRE-like 1 element, a possible novel kind of ERE, resulting in the upregulation of γ-GCS and GRX, and thereby preventing myocardial cell apoptosis under oxidative stress [38].

Fig. 2 — Schematics of hypothetical model of E2/ERα-enhanced calcineurin protein degradation by PI3 K/Akt/MDM2 signaling activation, contributing to inhibition of ISO-induced myocardial cell apoptosis. [25]; Bad = Bcl-2-associated death promoter protein; Cyt C. = cytochrome C; ISO = isoproterenol. For abbreviations, see text.
GPR30 protects against myocardial ischemia-reperfusion injury through activation of the PI3 K/Akt pathway [39]. Consequently, we conclude that all three ER subtypes contribute to E2-mediated cardioprotective effects against ischemia-reperfusion injury through activation of the PI3 K-nongenomic pathway, which leads to the activation of NOS/nitric oxide signaling in cardiomyocytes.

In addition to ROS, hypoxia-induced BNIP3, a Bcl-2 family of pro-apoptotic proteins comprising a subclass of SH3-only proteins, plays an important role in the development of hypoxia-induced cardiac hypertrophy and cardiomyocyte death [40]. We have recently found that E2 protects against BNIP3-induced apoptosis and that the protective role E2 plays might be governed by genomic and nongenomic effects. Our preliminary data suggest that ERα may bind to the regulatory region of the BNIP3 gene, which is probably located on the AP1 or NFκB binding site within the promoter, leading to the activation of NOS/nitric oxide signaling in cardiomyocytes.

Fig. 3 – Schematics of hypothetical model of E2 and ERα inhibiting myocardial cell hypertrophy by preventing cytosolic calcium accumulation through GSK-3β & I2PP2A activation, then inhibiting PP2A to activate PP1 and suppress the association between PP2A and NCX. [28]; PKA = Protein kinase A. For abbreviations, see text.
overexpression of ERs in cardiomyocytes can mitigate BNIP3-induced autophagy and apoptosis. However, we found that necrosis marker cTnT was significantly increased when autophagy was inhibited by 3-methyladenine. Further investigations are needed to clarify the effects of estrogen and its receptors on autophagy in cardiomyocytes.

7. E2/ERs protect against IGF2 receptor death signals in cardiomyocytes

The IGF 2 receptor (IGF2R), also called the cation-independent mannose-6-phosphate receptor, is a protein that in humans is encoded by the IGF2R gene. IGF2R is a multifunctional protein receptor that binds IGF2 at the cell surface and mannose-6-phosphate-tagged proteins in the trans-Golgi network [42]. Although IGF2R has been shown to clear IGF2 to attenuate signaling, the function of IGF2R in heart tissue is poorly understood.

Our previous studies found that activation of IGF2R in cardiomyocytes induced by hypertension, angiotensin II, and inomycin, as well as overexpression of IGF2R, not only leads to cardiomyocyte apoptosis through the Gαq—calcineurin pathway, but also contributes to MMP2/9 (matrix metalloproteinase) activation and myocardial extracellular matrix (ECM) remodeling [43–45]. These findings suggest that the suppression of IGF2R signaling pathways may be a good strategy to prevent the progression of pathological hypertrophy. Recently, we investigated the effects of E2 on IGF2R-activated myocardial cells and found that the activation of the PI3 K-Akt pathway by E2 markedly attenuated IGF2R-induced apoptosis and hypertrophy in cardiomyocytes [46]. These data indicate that E2 has protective effects against IGF2R-induced hypertrophy and cardiomyocyte death (Fig. 4). More research is necessary to characterize whether this protection is ER-dependent and to determine the precise mechanisms responsible for this cardioprotection.

8. Conclusions and future perspectives

ERα, ERβ, and GPR30 confer cardioprotective effects against various stresses by preventing myocardial cell apoptosis and cardiac hypertrophy. Our laboratory is investigating the possible regulation of autophagy by estrogen and ER-mediated cardioprotection. This might be the critical step to fully reveal the complex cellular mechanisms of estrogen and ER in cardiomyocytes, owing to the fact that the alternation in of cardiac basal autophagy (either an increase or a decrease) is demonstrably involved in various heart diseases, such as ischemic injury, cardiac hypertrophy, cardiac remodeling, and heart failure.

REFERENCES


[40] Weng YJ, Kuo WW, Tsai CH, Lin TE, Huang CY. Regulatory mechanism of 17β-estradiol and/or estrogen receptor β on hypoxia-induced BNIP3 autophagic and apoptotic pathways in H9c2 cardiomyoblast cells. First symposium on adaptive medicine and adaptability across the strait. Taipei, Taiwan: National Yang-Ming University; 2010 Sep 10–12.


Review article

Repair of bone defects with gelatin-based composites: A review

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Abstract

Numerous biomaterials are used in bone replacement therapy to repair defects caused by trauma, inflammation, tumor resection, or skeletal abnormalities. Ideally, the replacement material must be biocompatible and must be able to be reabsorbed or to dissolve naturally as the bone grows, yielding a newly remodeled bone. Gelatin, a partially denatured derivative of collagen, is biodegradable, exhibits good biocompatibility, and is less antigenic than collagen. Gelatin-based composites, therefore, provide an excellent scaffold for bone replacement. This paper provides a review of the work of the past decade in our laboratory on the development of gelatin-based composites that are suitable for repairing bone defects.

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1. Introduction

Bioactive ceramics, such as hydroxyapatite, tricalcium phosphate (TCP), and bioglass are widely applied in clinical settings to strengthen the biocompatibility and osteoconductive characteristics of bone replacements [1–3]. Granular TCP (Ca$_3$(PO$_4$)$_2$) is a biodegradable bone replacement material composed of calcium and phosphate ions that is commonly employed to repair bone defects. Although TCP can be easily packed into the bone defect without prior molding or shaping, granular TCP is difficult to maintain within the defect site and lacks structural stability.

Gelatin binds well to TCP, resulting in a composite material that has good biocompatibility, mechanical strength, and plasticity [4]. However, in vivo studies have shown that gelatin-TCP-composite materials are readily resorbed primarily due to dissolution and enzymatically catalyzed hydrolysis of the gelatin component of that composite material [5]. Various synthetic crosslinkers, such as formaldehyde, glutaraldehyde, polypeoxy compounds, tannic acid, dimethylsuberimidate, carbodiimides, and acyl azide have been used to crosslink gelatin to prolong the absorption of the gelatin in living tissue and improve the mechanical properties of the composites [6–8]. These synthetic crosslinkers are, however, highly cytotoxic, thereby reducing the biocompatibility of gelatin-based synthetically crosslinked implants. In our previous study, we used glutaraldehyde to fix a gelatin-TCP mixture (GTG) [9]. The in vitro cytotoxicity evaluation revealed that the concentration of glutaraldehyde solution used as the crosslinking agent in GTG composites should be lower than 8%. Additionally, GTG should be soaked in distilled water for at least 4 days to decrease its toxicity before clinical application.

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Bone tissue engineering has the potential to regenerate bone with natural form and function. Successful bone tissue engineering depends on a suitable source of cells, appropriate culture conditions, and a biocompatible scaffold with a highly porous and interconnected pore structure. Recent advances in regenerative medicine have shown that stem cells can play an important role in the repair of skeletal defects. Bone marrow stromal cells (BMSCs) are a promising cell source for the important role in the repair of skeletal defects. Bone marrow stromal cells are a promising cell source for the repair of skeletal defects and their favorable restoration of bone defects because of their relative ease of culture conditions, and a biocompatible scaffold with a highly porous and interconnected pore structure. Recent advances in regenerative medicine have shown that stem cells can play an important role in the repair of skeletal defects. Bone marrow stromal cells (BMSCs) are a promising cell source for the repair of skeletal defects.

2. Gelatin crosslinked with natural crosslinking reagents

Genipin is a natural crosslinking agent that is extracted from the fruits of Gardenia jasminoides Ellis. In our laboratory, we developed a material composed of genipin-crosslinked gelatin combined with TCP ceramic particles (GGT) that could be used as a scaffold for filling bone defects. We found that the degree of crosslinking and the rate of in vitro degradation of genipin-crosslinked gelatin could be controlled by varying the concentration of genipin. The concentration of genipin for more complete crosslinking reaction in the GGT composite was 0.5 wt% [17,18]. However, the concentration of genipin must be greater than 0.5 wt% to eliminate the risk of cytotoxicity [19]. In fact, we found that a concentration of genipin that exceeds 80 ppm in the culture medium was cytotoxic to osteoblasts [18]. Experiments on the subcutaneous implantation of the GGT composite in rats demonstrated that the foreign body capsule of the composite that had been crosslinked with 1.00 wt% genipin was much thicker than that of composites that had been crosslinked with 0.05, 0.10 or 0.50 wt% genipin. In addition, the composite had a low degree of porosity (68.0% ± 2.5%) after adding genipin. Therefore, low-toxicity crosslinkers are required to enable implants to form stable and biocompatible crosslinked materials without inducing a cytotoxic effect.

Oligomeric proanthocyanidins (OPCs) are naturally occurring, minimally cytotoxic crosslinkers that are present in many fruits, vegetables, nuts, seeds, and flowers as well as in the bark of many plant species. OPCs belong to a subgroup of flavonoid-condensed tannins, which are highly hydroxylated structures that can form insoluble complexes with carbohydrates and proteins [20]. These proanthocyanidins have been successfully used to fix biologic tissues and biomaterials, including porcine valves, collagen matrices, and chitosan-gelatin films and have been shown to be minimally cytotoxic [21–24].

We prepared a biodegradable GTP composite comprising an OPC-crosslinked gelatin mixed with TCP for use as a bone substitute [25,26]. When 5.0 wt% of OPC was added, the crosslinking reaction between gelatin and OPCs proceeded as close to completion as possible. The result indicated that the ability to crosslink gelatin molecules was higher in genipin than in OPCs. However, adding 5 wt% of OPCs to composites was effective at providing resistance to degradation in vitro and in vivo. Moreover, OPCs at a concentration of less than 100 ppm were able to promote the proliferation of MG-63 cells, indicating that genipin has higher toxicity than OPCs. Additionally, evaluation of cytotoxicity demonstrated that OPCs, gelatin, and calcium ion gradually released from the GTP composite facilitated the growth of MG-63 cells in vitro. The results of the in vivo evaluation of the subcutaneous implantation of the composite material in rats revealed a fairly uniform layer of surrounding fibrous tissue. There was no variation in the thickness of foreign body capsules of the GTP composites crosslinked with OPCs at a wt% concentration of 5.0, 7.5 or 10.0 wt%. Furthermore, experiments on the biological response of the GTP composite using a rabbit calvarial defect model demonstrated that the GTP composite did not cause any deleterious effects on the underlying brain tissues. Moreover, there was more evidence of new bone formation in the group that received a GTP composite comprising 5.0 wt% of OPCs than in the group that received deproteinized bovine bone over all implantation periods. Progressive replacement of the GTP composite by new bone was preceded by a combination of osteoconduction and biodegradation.

3. BMSCs loaded onto a porous gelatin-based composite scaffold

The excellent biocompatibility and osteoconductivity of the GTP composites make them promising materials for the clinical repair of bone defects. However, GTP composites have a dense morphology after the addition of OPCs. The dense morphology will impede the supply of oxygen and nutrients to the attached cells inside the scaffold and will detrimentally affect cell ingrowth. On the contrary, high porosity, large pores, and a three-dimensionally interconnected pore structure in the scaffold will provide sufficient space for the ingrowth of both new bone tissue and blood vessels following implantation into the host tissue.

We prepared a macroporous GTP composite containing TCP and OPCs crosslinked with gelatin using a salt-leaching method and found that the amount of salt added strongly influenced the morphology of the GTP composite. The GTP composite had a relatively homogeneous pore structure and a high porosity (~73%) when the weight ratio of salt particulates to composite was 4:1. The macropore sizes ranged from 400 μm to 550 μm. The cytotoxicity assay demonstrated that the extract from the porous GTP composite enhanced the proliferation of rat BMSCs and that the enhanced proliferation was attributable to the release of gelatin, OPCs, and calcium. In another experiment, we combined BMSCs with a porous GTP composite as a scaffold for bone tissue engineering. Rat BMSCs were seeded onto the porous GTP composite and cultured in a spinner flask to improve nutrient supply and metabolite removal. After 2 weeks of dynamic culturing, we found that the cells penetrated the pores and proliferated on the OPC-crosslinked gelatin mixed with TCP for use as a bone substitute [25,26]. When 5.0 wt% of OPC was added, the crosslinking reaction between gelatin and OPCs proceeded as close to completion as possible. The result indicated that the ability to crosslink gelatin molecules was higher in genipin than in OPCs. However, adding 5 wt% of OPCs to composites was effective at providing resistance to degradation in vitro and in vivo. Moreover, OPCs at a concentration of less than 100 ppm were able to promote the proliferation of MG-63 cells, indicating that genipin has higher toxicity than OPCs. Additionally, evaluation of cytotoxicity demonstrated that OPCs, gelatin, and calcium ion gradually released from the GTP composite facilitated the growth of MG-63 cells in vitro. The results of the in vivo evaluation of the subcutaneous implantation of the composite material in rats revealed a fairly uniform layer of surrounding fibrous tissue. There was no variation in the thickness of foreign body capsules of the GTP composites crosslinked with OPCs at a wt% concentration of 5.0, 7.5 or 10.0 wt%. Furthermore, experiments on the biological response of the GTP composite using a rabbit calvarial defect model demonstrated that the GTP composite did not cause any deleterious effects on the underlying brain tissues. Moreover, there was more evidence of new bone formation in the group that received a GTP composite comprising 5.0 wt% of OPCs than in the group that received deproteinized bovine bone over all implantation periods. Progressive replacement of the GTP composite by new bone was preceded by a combination of osteoconduction and biodegradation.
the scaffolds, indicating that the porous GTP scaffold is an appropriate material for the ingrowth of cells. We also tested whether the GTP scaffold could promote the ingrowth of new blood vessels from neighboring host tissues. The defect cavity in rats was filled with a BMSC-seeded GTP scaffold. We found that numerous erythrocytes were present in the BMSC-seeded scaffold at Week 4, suggesting that the interconnected macropores in the GTP scaffold promoted the ingrowth of new blood vessels from neighboring host tissues. We also found that there were numerous cells around the pores in the BMSC-seeded GTP scaffold. The cells that differentiated into bone-forming osteoblasts were probably derived from the seeded BMSCs. These results indicate that the seeded BMSCs, the postrepair vascularization, and the release of gelatin, calcium, and OPCs from the scaffold are possible causes of the abundant proliferation of the cells at the cranial bone defect. These regenerating cells might modulate further development of bone tissue. After 8 weeks of implantation, the BMSC-seeded scaffold promoted more new bone formation than the acellular scaffold, suggesting that the use of BMSCs reduces the time required for the cells to invade the defect site.

4. Gelatin-based composites containing traditional Chinese herbal medicine

Many traditional Chinese herbal medicines are commonly used to treat orthopedic disorders and have been proven to be effective for bone regeneration [27]. We fabricated a composite composed of gelatin, TCP, genipin, and Chi-Li-Saan, a Chinese medicinal remedy, as a bone substitute to fill a large defect in the calvarial bone in rabbits [28]. The results revealed that animals that received the Chi-Li-Saan composite demonstrated a significantly greater amount of new bone growth than animals with bone defects that were not subjected to reconstruction therapy. In another study, we evaluated the activity of rat bone cells in animals that had been exposed to five different Chinese herbal drugs [29]. The results of bone cell culture experiments revealed that Cuscuta chinensis Lam. enhanced the proliferation and differentiation of osteoblasts but inhibited the activity of osteoclasts. Loranthus parasiticus Merr. and Achyranthes bidentata Bl. produced opposite results. Eucommia ulmoides Oliv. and Dipsacus asper Wall. promoted the proliferation and differentiation of osteoblasts but did not affect the activities of osteoclasts. The results of neonatal rat calvarias organ culture that had been exposed to a mixture of GGT composite and traditional Chinese medicine, such as Cuscuta chinensis Lam., Eucommia ulmoides Oliv. and Dipsacus asper Wall., demonstrated that these Chinese medicinal herbs effectively promoted the regeneration of defective bone tissue.

The dried rhizome of perennial pteridophyte Drynaria fortunei (Kunze) J. Sm., also known as Gu-Sui-Bu (GSB), is widely used in Asia for the treatment of bone-related diseases, including bone fracture, osteoporosis, and arthritis, and has been shown to have therapeutic effects on bone healing [27]. Naringin, a polymethoxylated flavonoid, is reportedly the main effective component of GSB, and has been shown to increase the amount of bone morphogenetic protein in osteoblasts. Several studies have demonstrated that GSB can enhance the proliferation and differentiation of osteoblasts as well as bone cell activities in vitro, and that it can inhibit the formation of osteoclasts [30-32]. We have found that GSB at a concentration of 100 μg/mL leads to a significant increase in osteoblast numbers, intracellular alkaline phosphatase levels, and nodule numbers, without influencing osteoclast activity [33]. Moreover, we have found that addition of GSB to GGT composites accelerates the regeneration of defective bone tissue.

We hypothesized that a combination of osteoinductive agents and BMSCs would promote bone healing. We, therefore, prepared a macroporous GGT composite using a salt-leaching method to carry GSB (GGT-GSB). The composite had a homogeneous pore structure with pore sizes ranging from 280 μm to 430 μm and a high porosity (~80%). Rabbit BMSCs were then seeded onto the porous composites. After a week of culture in a spinner flask, the cells effectively entered the scaffold. They were then autotransplanted into critical size calvarial defects in rabbits. After 8 weeks of implantation, new blood vessels formed and many erythrocytes were present in the BMSC-seeded GGT-GSB scaffold, indicating that blood vessels from the neighboring host tissues had successfully invaded the scaffold. There were also many regenerating osteoblasts in the peripheral and central areas of the autologous BMSC-seeded scaffold. These cells were probably derived from the seeded autologous BMSCs. Furthermore, the autologous BMSC-loaded GGT-GSB scaffold promoted more new bone formation at the defect site than the BMSC-seeded GGT and acellular scaffolds. In addition, new bone replaced a significant amount of GGT-GSB scaffold, revealing that the autologous BMSCs were responsible for bone formation at their locations. Moreover, GSB was gradually released from the biodegradable scaffold, which was most likely due to the effect of bone regeneration. Therefore, GSB can induce the formation of new bone by providing an effective biodegradable delivery system. Accordingly, a porous GGT scaffold composed of a GSB-based and autologous BMSC-based composite is an ideal biomaterial for the generation of new bone.

5. Conclusion

Bone tissue engineering is effective at repairing damaged or diseased skeletal tissue. A combination of gelatin with TCP can be used to create a biocompatible scaffold with osteoconductivity characteristics. The addition of genipin and OPCs, naturally occurring low-cytotoxic crosslinkers, effectively reduces the degradation rate of the gelatin-TCP mixture. Moreover, incorporating BMSCs, GSB, and Chinese medicinal extracts into a porous gelatin-TCP scaffold can accelerate bone regeneration.

REFERENCES


Review article

Effects of electrical stimulation on peripheral nerve regeneration

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ABSTRACT

Over the past few years, my group has been investigating the effects of different parameters of electrical stimulation on nerve regeneration of a 10-mm gap of rat sciatic nerve created between the proximal and distal nerve stumps, which were sutured into silicone rubber chambers. In this review, I will introduce our work and share our experience with investigators who are interested in the fields of nerve regeneration and biomedical engineering.

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1. Introduction

End-to-end and fascicular suture repair techniques are recommended for short nerve injury. However, in cases of extensive nerve injury, i.e., defects involving an irreducible gap between the injured proximal and distal stumps, a nerve graft or a nerve bridge is preferred. Donor nerves for grafting are often difficult to acquire; therefore, considerable research has been conducted on peripheral nerve repair using the nerve bridge technique [1–3]. A nerve bridge technique involves placing both ends of the injured nerve stumps into a tubular chamber, which helps guide growing nerve fibers along appropriate paths and enhances the precision of stump approximation.Regeneration of longer gaps can be achieved by prefilling the guidance chamber with chemical adjuncts such as neurite-promoting factors and neurotrophic factors, which can promote early peripheral nerve regeneration [4–6]. In addition to chemical adjuncts, physical adjuncts such as electrical stimulation (ES) have been used to recover lost function of injured nerve pathways in the peripheral nervous system.

Studies have demonstrated that a weak electric field can enhance neurite outgrowth in vitro [7,8] and in vivo [9,10]. Other studies, however, have reported that electric fields have no effect and in some cases a negative effect on nerve regeneration [11,12]. Similarly, discrepant findings have also been noted between studies that have adopted different stimulation frequencies and intensities. For example, Cheng et al found that pulse ES at 100 Hz could induce a relatively higher regenerated axonal density than electrical stimulation at 50 Hz [13]. However, Agnew et al found a positive correlation between the frequency of ES applied to a peripheral nerve and the severity of stimulation-induced neural damage [14]. In addition, the ideal duration that ES should be applied in patients with and in animal models of nerve injury has not been established. Furthermore, all of the aforementioned
studies that investigated the effect of ES on nerve regeneration in animal models focused on short nerve gaps. The inherent regenerative capacity of the nerve in animals could be so efficient over shorter gaps that the effects of ES may not be fully revealed. Therefore, animal models of nerve injury involving longer nerve gaps are needed to better understand the effects of ES on damaged nerves.

This review will introduce the effects of different frequencies, current intensities, and durations of ES on the regeneration of transected rat sciatic nerves that were reconnected using a silicone rubber nerve tube with a 10-mm gap.

2. Effects of ES frequency on sciatic nerve regeneration

It has been reported that ES can enhance peripheral nerve regeneration [15,16]. Based on the results of those studies, we are confident that ES produces bio-effects on nerve tissues. However, contradictory results have also been reported. Therefore, a dramatic and reproducible model with well-controlled experimental variations is necessary to clarify the role that electrical treatment plays on nerve regeneration.

The nature of the experiments, such as the type of ES used (DC or AC; constant or pulsed), the stimulation parameters, the sites for the placement of electrodes, and most importantly the length of the nerve gap, all affect the efficacy of ES on nerve regeneration. Considerable research has been conducted on nerve repair across a wide gap using entubulation techniques [17]; however, to the best of our knowledge, Cheng et al are the only researchers to have used conduit prostheses to investigate the influence of different ES frequencies on nerve regeneration [13]. In their study, histomorphometric evaluation revealed that ES frequency affected nerve fiber density. Unfortunately, the small nerve defect (7 mm in length) and the lack of electrophysiologic data hindered their ability to make a solid conclusion.

In a recent study, we used a silicone rubber conduit to repair a rat sciatic nerve defect measuring 10 mm in length and then stimulated the nerve with different electrical frequencies. Histological and electrophysiological techniques were used to determine whether ES could stimulate the regeneration of nerves. We found that ES significantly suppressed the formation of nerve cables across the nerve gap in the silicone rubber chamber in a dose-dependent manner. Our data showed that a frequency of 2 Hz resulted in generation of nerve cables across the gap in 86% of the subjects, that a frequency of 20 Hz stimulated nerve regeneration in 71% of the animals, and that 200 Hz resulted in regeneration of nerve cables across the gap in only 57% of the test subjects. In contrast, bridging cables were noted in all of the animals in the control group as well as in the ES group that received ES at a frequency of 1 Hz. These findings show that electrical treatment may interfere with the process of nerve regeneration. However, examination of muscle action potentials (MAPs) and morphology revealed that ES seems to exert a growth-promoting effect on regenerated nerves. Morphometric studies revealed that the regenerated nerves that received electrical treatment at a frequency of 2 Hz had a significantly shorter latency, a longer duration, a faster nerve conductive velocity (NCV), a smaller cross-sectional area, a larger axonal density, and a larger ratio of blood vessel area to total nerve than controls. Those findings indicate that electrical treatment can accelerate the maturation of regenerated nerves [18].

These results raise a number of questions. For example, how can the discrepant results be explained? In addition, how should “successful nerve regeneration” within a guidance tube be defined? We believe that both the percentage of regenerated nerves that successfully cross the gap as well as the maturity of nerve microstructure must be considered when assessing the recovery of regenerated nerves.

3. Effects of ES current intensity on sciatic nerve regeneration

Several investigators have tried to explain how application of cathode distal current enhances the regeneration of peripheral nerves. Sisken et al reported that direct current resulted in an increased number of neurotrophic factor receptors in chick embryos [19]. It has also been reported that proteoglycan-mediated adhesion of regenerating axons, which is necessary for neuronal cell growth, could be manipulated by direct current [20]. In addition, some studies showed that treatment with ES led to an increase in the expression of injury/regeneration-associated genes (growth-associated protein 43 and Tβ1 tubulin) as well as neurotrophin brain-derived neurotrophic factor and its receptor trkB, factors that play important roles in the regeneration of nerve tissues [15,16].

We also found that animals exposed to ES had a larger mean number of axons, endoneurial area, total nerve area, blood vessel number, and blood vessel area than control animals, which indicates that ES accelerates the maturation of regenerated nerves [21]. In addition, regenerated nerves treated with ES, especially in the group that received 1 mA of direct current, had relatively shorter latency periods, larger amplitudes, larger MAP areas, faster NCVs, and more evidence of reinnervation of muscle fibers than controls. These results indicate that the transected nerves that received ES underwent adequate regeneration. We also found that the ability of ES to improve the function of regenerated nerves decreased as the current intensity increased. For example, animals that received ES at 4 mA had a significantly higher error rate than controls while crossing the grid runway in the kinematic gait analysis, a test that assesses individual limb motor functions [22]. Our results indicate, therefore, that excessive ES can hinder the functional recovery of regenerated nerves.

4. Effects of ES timing on sciatic nerve regeneration

It is generally assumed that ES should be applied shortly after nerve injury because ES-induced recovery can be facilitated by the body’s immune response to injury [23,24]. In addition, studies have shown that ES can also accelerate upregulation of brain-derived neurotrophic factor (BDNF) and trkB mRNA,
factors that support the development, maintenance, and plasticity of peripheral neurons [15,16]. Accordingly, delayed onset of ES should be less effective in promoting the recovery of regenerated nerves. However, those studies assessed ES only over a short gap. We wondered whether more severely injured animals require more time before ES can increase neuroplasticity. In one of our current studies [25], we found that the number of axons was significantly greater in rats that received ES at a frequency of 2 Hz and an intensity of 1 mA on Day 8 following nerve repair than in rats that did not receive ES. This result confirms that ES accelerates the maturation of regenerated nerves that successfully cross the gap and leads to improved sensorimotor function after peripheral nerve injury. In addition, we also found that application of a delayed ES could dramatically improve the recovery of regenerated nerve function. This beneficial effect was not seen when the same stimulation protocol was applied immediately after nerve repair. Specifically, we found that a delay of 7 days before the onset of ES significantly enhanced the formation of nerve cables across a wide nerve gap in the silicone rubber chamber. Seventy percent of the animals in that group had cables that grew across the gap whereas only 30% of the animals in the group that received ES on post-injury Day (PID) 1 exhibited such bridging cables. Nerve recovery after a delayed onset of ES was a surprising finding, as most studies have shown that early application of ES is effective at accelerating axonal regeneration, mainly by up-regulating the expression of growth-promoting factors such as BDNF [10,26]. However, in those studies the nerve gap was much shorter than that in our study. Therefore, we do not know whether more severely injured animals require more time after nerve repair before ES can increase neuroplasticity. In addition, when considering the onset of ES following nerve repair, it has to be kept in mind that early application of ES might result in side effects, such as exacerbation of the size of the lesion [27]. For example, Griesbach et al found that BDNF levels significantly increased in rats with a mild fluid-percussion injury (FPI) that were exercised from PID 14 to 20. In rats with moderate FPI, however, significant increases in BDNF were evident only in animals that were exercised from PID 30 to 36 [28]. Those results indicate that the time window for exercise-induced increases in BDNF is dependent on injury severity. Since both ES and exercise share some common mechanisms of action (i.e. increased expression of neurotrophic BDNF and its receptor trkB) [29], it is reasonable to assume that delayed ES should be more effective than immediate ES in promoting nerve regeneration across a large gap. Interestingly, we only found significant improvement in the rate of successful regeneration when ES was started after a delay of 7 days, but not when performed between Days 15 and 29 post-injury. This result again indicates the importance of the timing phenomenon for the effect of ES on growth-promoting factors [30].

It is also important to note that delayed ES not only increased the rate of successful regeneration, but also enhanced maturity of the neural components within the nerve cable [25]. Specifically, the number of myelinated axons that successfully grew across the 10-mm gap was twofold greater in the groups that received delayed ES than in the group that received immediate ES. In addition, the number of regenerated blood vessels was greater and the nerve areas were larger in the groups that received delayed ES than in the group that received immediate ES. Although we cannot explain the increased number of regenerated axons and blood vessels, our results indicate that delayed application of ES affects axonal and capillary growth in the regenerated nerves.

Our findings substantiate that the time course of ES is of importance for the final recovery after peripheral nerve injuries. A short delay in the onset of ES to injured nerves can significantly accelerate axonal regrowth and functional restoration, which are important factors for successful nerve regeneration.

5. Conclusion

This review demonstrates that ES has a dual effect: it can hinder the growth of regenerating nerves as well as promote their recovery. Safe stimulus protocols, therefore, are necessary. Otherwise, improper ES can irreversibly damage nerve tissue, retarding the process of nerve regeneration.

REFERENCES

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These are short discussions of a case or case series with unique features not previously described that make an important teaching point or scientific observation. They may describe novel techniques, novel use of equipment, or new information on diseases of importance. Section headings should be: Abstract, Introduction, Case Report, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

The Introduction should describe the purpose of the report, the significance of the disease and its specificity, and briefly review the relevant literature.

The Case Report should include the general data of the case, medical history, family history, chief complaint, present illness, clinical manifestation, methods of diagnosis and treatment, and outcome.

The Discussion should compare, analyze and discuss the similarities and differences between the reported case and similar previously reported cases. The importance or specificity of the case should be restated when discussing the differential diagnoses. Suggest the prognosis of the disease and possibility of prevention. Typical length: no more than 1500 words, 20–40 references.

8.4. Short Communications
These should be concise presentations of clinical or preliminary experimental results. Section headings should be: Abstract, Introduction, Methods, Results, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

Typical length: no more than 1000 words, 20–40 references, with no more than four figures or tables. The Editors reserve the right to decide what constitutes a Short Communication.

8.5. Letters to the Editor
Letters are welcome in response to previously published articles, and may also include interesting cases that do not meet the requirement of being truly exceptional, as well as other communications of general interest. Letters should have a title and include appropriate references, and include the corresponding author’s mailing and e-mail addresses. Letters are edited, sometimes extensively, to sharpen their focus. They may be sent for peer review at the discretion of the Editors. Letters are selected based on clarity, significance, and space. Typical length: no more than 600 words, 5–10 references; 1 table and/or 1 figure may be included.

8.6. Editorials
Editorials are invited articles or comments concerning a specific paper in the Journal or a topical issue in the field. While normally invited, unsolicited editorials may be submitted. Typical length: no more than 1500 words, 15–30 references.

9. Manuscript Preparation
Text should be typed double-spaced on one side of white A4 (297 × 210 mm) paper, with outer margins of 2.5 cm. A manuscript should include a title page, abstract, text, acknowledgments (if applicable), conflicts of interest statement (if any), references, and figures and tables as appropriate. Each section of the manuscript should begin on a new page. Pages should be numbered consecutively, beginning with the title page.

9.1. Title Page
The title page should contain the following information (in order, from the top to bottom of the page):
- category of paper
- article title
- names (spelled out in full)* of all the authors, and the institutions with which they are affiliated; indicate all affiliations with a superscripted lowercase letter after the author’s name and in front of the appropriate affiliation
- corresponding author details (name, e-mail, mailing address, telephone and fax numbers)

*The name of each author should be written with the family name last, e.g., Jing-Lin Chang. Authorship is restricted only to direct participants who have contributed significantly to the work.

9.2. Abstract and Keywords
Abstracts should be no more than 300 words in length. Abstracts for Original Articles should be structured, with the section headings: Background/Introduction, Purpose(s)/Aim(s), Methods, Results, Conclusion. Abstracts for Case Reports are unstructured, but should include the significance and purpose of the case presentation, the diagnostic methods of the case, the key data, and brief comments and suggestions with regard to the case. Abstracts for Review Articles and Short Communications should also be unstructured. No abstract is required for Letters to the Editor and Editorials. For the article categories that require an abstract, 3–5 relevant keywords should also be provided in alphabetical order.
9.3. Main Text
The text for Original Articles should be organized into the following sections: Background/Introduction, Purpose(s)/Aim(s), Methods, Results and Discussion. Sections for Case Reports are: Introduction, Case Report, and Discussion. Each section should begin on a new page.

9.3.1. Abbreviations
Where a term/definition will be continually referred to, it must be written in full when it first appears in the text, followed by the subsequent abbreviation in parentheses. Thereafter, the abbreviation may be used. An abbreviation should not be first defined in any section heading; if an abbreviation has previously been defined in the text, then the abbreviation may be used in a subsequent section heading. Restrict the number of abbreviations to those that are absolutely necessary.

9.3.2. Units
Système International (SI) units must be used, with the exception of blood pressure values which are to be reported in mmHg. Please use the metric system for the expression of length, area, mass, and volume. Temperatures are to be given in degrees Celsius.

9.3.3. Names of drugs, devices and other products
Use the Recommended International Non-proprietary Name for medicinal substances, unless the specific trade name of a drug is directly relevant to the discussion. For devices and other products, the generic term should be used, unless the specific trade name is directly relevant to the discussion. If the trade name is given, then the manufacturer name and the city, state and country location of the manufacturer must be provided the first time it is mentioned in the text, for example, “...SPSS version 11 was used (SPSS Inc., Chicago, IL, USA).”

9.3.4. Statistical requirements
Statistical analysis is essential for all research papers except case reports. Use correct nomenclature of statistical methods (e.g., two sample t test, not unpaired t test). Descriptive statistics should follow the scales used in data description. Inferential statistics are important for interpreting results and should be described in detail.

All p values should be expressed to 2 digits to the right of the decimal point, unless p < 0.01, in which case the p value should be expressed to 3 digits to the right of the decimal point. The smallest p value that should be expressed is p < 0.001, since additional zeros do not convey useful information; the largest p value that should be expressed is p > 0.99.

9.3.5. Personal communications and unpublished data
These sources cannot be included in the references list but may be described in the text. The author(s) must give the full name and highest academic degree of the person, the date of the communication, and indicate whether it was in oral or written (letter, fax, e-mail) form. A signed statement of permission should be included from each person identified as a source of information in a personal communication or as a source for unpublished data.

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General acknowledgments for consultations, statistical analysis, etc., should be listed concisely at the end of the text, including the names of the individuals who were directly involved. Consent should be obtained from those individuals before their names are listed in this section. All financial and material support for the research and work from internal or external agencies, including commercial companies, should be clearly and completely identified. Ensure that any conflicts of interest (financial and/or non-financial) are explicitly declared.

9.5. References

9.5.1. In the main text, tables, figure legends
• References should be indicated by numbers in square brackets in line with the text, and numbered consecutively in order of appearance in the text.
• References cited in tables or figure legends should be included in sequence at the point where the table or figure is first mentioned in the main text.
• Do not cite uncompleted work or work that has not yet been accepted for publication (i.e., “unpublished observation”, “personal communication”) as references. Also see Section 9.3.5. above.
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9.5.2. In the references section
• References should be limited to those cited in the text and listed in numerical order, NOT alphabetical order.
• References should include, in order, author surnames and initials, article title, abbreviated journal name, year, volume and inclusive page numbers. The last names and initials of all the authors up to 6 should be included, but when authors number 7 or more, list the first 6 authors only followed by “et al”. Abbreviations for journal names should conform to those used in MEDLINE.
• If citing a website, provide the author information, article title, website address and the date you accessed the information.
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Authors are responsible for the accuracy and completeness of their references and for correct text citation.

Examples are given below.

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**Journal supplement**

**Journal article not in English but with English abstract**

**Book**

**Book chapter in book with editor and edition**

**Bulletin**

**Company/manufacturer publication/pamphlet**

**Electronic publications**


**Items presented at a meeting but not yet published**

Greenspan A, Eerdekens M, Mahmoud R. Is there an increased rate of cerebrovascular events among dementia patients? Poster presented at: 24th Congress of the Collegium Internationale Neuro-Psychopharmacologicum (CINP); June 20–24, 2004; Paris, France.


**Item presented at a meeting and published**

**Material accepted for publication but not yet published**


**Theses and dissertations**


**Website**

### 9.6. Tables
Tables should supplement, not duplicate, the text. They should have a concise table heading, be self-explanatory, and numbered consecutively in the order of their citation in the text. Information requiring explanatory footnotes should be denoted using superscripted lowercase letters in alphabetical order (a, b, c, etc.). Asterisks (*, **) are used only to indicate the probability level of tests of significance. Abbreviations used in the table must be defined and placed after the footnotes. If you include a block of data or table from another source, whether published or unpublished, you must acknowledge the original source.
9.7. Figures

9.7.1. General guidelines
The number of figures should be restricted to the minimum necessary to support the textual material. They should have an informative figure legend and be numbered in the order of their citation in the text. All symbols and abbreviations should be defined in the legend. Patient identification should be obscured. All lettering should be done professionally and should be in proportion to the drawing, graph or photograph. Photomicrographs must include an internal scale marker, and the legend should state the type of specimen, original magnification and stain.

Figures must be submitted as separate picture files at the correct resolution (see Section 9.7.2. below). The files should be named according to the figure number, e.g., “Article1_Fig1”, “Article1_Fig2”.

9.7.2. Formats
Regardless of the application used, when your electronic artwork is finalized, please “save as” or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

- EPS: Vector drawings. Embed the font or save the text as “graphics”.
- TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
- TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
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Please do not:

- Supply files that are optimized for screen use (like GIF, BMP, PICT, WPG); the resolution is too low;
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As a general rule, the receipt of a manuscript will be acknowledged within 1 week of submission, and authors will be provided with a manuscript reference number for future correspondence. If such an acknowledgment is not received in a reasonable period of time, the author should contact the Editorial Office.

Submissions are reviewed by the Editorial Office to ensure that it contains all parts. The Editorial Office will not accept a submission if the author has not supplied all the material and documents as outlined in these author instructions.

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Once a manuscript has been accepted for publication, the authors should submit the final version of the manuscript in MS Word format, with all tables/figures as applicable, to the Editorial Office.

Accepted manuscripts are copyedited according to the Journal’s style and PDF page proofs are e-mailed by the Publisher to the corresponding author for final approval. Authors are responsible for all statements made in their work, including changes made by the copy editor.

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