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G.　知的所有権の取得状況

1) 特許取得

なし

2) 実用新案登録

なし

3) その他

なし

研究成果の刊行に関する一覧表

著書

なし

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Review

Roles of prostaglandin E2 in the cochlea

Takayuki Nakagawa*

Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyoku, Kyoto 606-8507, Japan

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ABSTRACT

Prostaglandins are one of the major groups of chemical mediators in the mammalian body. Among prostaglandins, prostaglandin E2 (PGE2) is the most abundant prostanoid in humans and involved in regulating many different fundamental biological functions. PGE2 signaling is mediated by four distinct E-prostanoid receptors (EPs) namely EP1–4. Recently, accumulating evidence indicates critical, but complex roles of EP signaling in the pathogenesis of neuronal diseases depending on the context of neuronal injury. Four distinct EPs are expressed in the stria vascularis, spiral ligament, spiral ganglion and organ of Corti, indicating an involvement of EP signaling in the cochlear function. Activation of EP4 in cochleae significantly attenuates noise-induced damage in cochleae, and activation of EP2 or EP4 induces the formation of vascular endothelial growth factor in cochleae. These findings strongly suggest that individual EP signaling may be involved in the maintenance of the cochlear sensory system similarly to the central nervous system. This review highlights recent findings on EP signaling in the central nervous system, and presents its possible roles in regulation of blood flow, protection of sensory cells and immune responses in cochleae.

1. Introduction

Prostaglandins (PGs), one of the major groups of chemical mediators in the mammalian body, are involved in numerous physiological reactions. Prostaglandin E2 (PGE2) is the most abundant prostanoid in humans and involved in regulating many different fundamental biological functions (Legler et al., 2010). Accumulating evidence indicates critical roles of PGE2 signaling in the pathogenesis of neuronal diseases (Andreasson, 2010). However, the role of PGE2 in neuronal diseases is complex. The divergent effects of PGE2 signaling likely depend on distinct patterns and dynamics of expression of PGE2 receptors, namely E-prostanoid receptor (EP) 1–4 (Sugimoto and Narumiya, 2007). In the central nervous system, some EP signaling pathways mediate neurotoxic effects, but others appear to mediate paradoxically protective effects (Andreasson, 2010). Therefore, EP signaling pathways have gained particular attention as new targets in therapeutics in neuronal diseases.

Sensorineural hearing loss (SNHL) is one of the most common disabilities, and has limited therapeutic options. SNHL can be divided into two types corresponding to its onset, gradual or acute onset. The most prevalent SNHL with gradual onset is age-related hearing loss. Typical SNHL with acute onset is sudden sensorineural hearing loss (SSHL). Therapeutic options for SNHL with gradual onset are limited to hearing aids or cochlear implants, while pharmacotherapeutic approaches are sometimes effective for SNHL with acute onset. Systemic steroid application has been a primary choice for treatment. However, 20% of patients with SSHL are resistant to systemic steroid treatment. Hence, additional pharmacotherapeutic options for SSHL have been required. Prostaglandin E1 (PGE1), which is a dihomo-g-linolenic acid derived eicosanoid, has long been used as a vasodilator clinically, and proven to be effective on diverse circulatory disorders. Disorders of cochlear blood flow have been considered one of principle causes for SSHL (Nakashima et al., 2003), which is a reason for clinical use of several vasodilators including PGE1 in treatment of SSHL. In fact, PGE1 has often been used as a secondary choice of therapeutic options for SSHL. However, its clinical benefit is still controversial (Nakashima et al., 1989; Ogawa et al., 2002; Ahn et al., 2005; Zhuo et al., 2008; Agarwal and Pothier, 2009). PGE1 mainly binds to EPs, and show various biological effects including vasodilation. Therefore, activation or inhibition of specific EPs could boost therapeutic potential of PGE1 or PGE2 (Andreasson, 2010; Legler et al., 2010;

Abbreviations: AC, adenylyl cyclase; AA, arachidonic acid; cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; DAG, diacylglycerol; EP, E-prostanoid receptor; IP3, inositol-1,4,5-trisphosphate; MRP4, multidrug resistance protein-4; PLC, phospholipase C; PGs, prostaglandins; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PGE1, prostaglandin E1; PGES, prostaglandin-E-synthase; PGF2α, prostaglandin F2α; PGH2, prostaglandin H2; PKA, protein kinase A; SNHL, sensorineural hearing loss; SSHL, sudden sensorineural hearing loss.

* Tel.: +81 75 751 3346; fax: +81 75 751 7225.
E-mail address: tnakagawa@ent.kuhp.kyoto-u.ac.jp.

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Sugimoto and Narumiya, 2007). However, little is known about EPs in the cochlea. This review presents recent findings on EPs in the cochlea with an overview of their roles in neurological disorders.

2. PGE2 biosynthesis and metabolism

PGs are short-lived potent bioactive lipid messengers belonging to the family of eicosanoids (Funk, 2001; Harris et al., 2002; Simmons et al., 2004; Smith et al., 2000). PGs derive from 20-carbon fatty acid precursors, mainly arachidonic acid (AA). PG biosynthesis is controlled by the rate-limited release of AA, obtained either from plasma membrane phospholipids or derived by desaturation and elongation of dietary fatty acids such as linoleic acid (Simmons et al., 2004; Legler et al., 2010). This process is mediated by phospholipase A2, following activation of intracellular signal transduction pathways. Following its release from intracellular stores into the cytoplasm, AA is oxidized by cyclooxygenase (COX) to prostaglandin H2 (PGH2). This intermediate serves as the substrate for terminal prostanoid synthase enzymes. These are named according to the prostaglandin they produce such that prostaglandin D2 (PGD2) is synthesized by prostaglandin-D-synthase, PGE2 by prostaglandin-E-synthase (PGES); prostaglandin F2α (PGF2α) by prostaglandin-F-synthase, prostacyclin by prostaglandin-I-synthase and thromboxane by thromboxane synthase (Legler et al., 2010; Park et al., 2006) (Fig. 1).

Most cells synthesize almost undetectable or basal levels of PGs. PGs are de novo synthesized rapidly upon cell activation and act in an autocrine and paracrine fashion. A variety of stimuli regulate the synthesis of PGs, which have an extraordinary broad spectrum of action (Funk, 2001; Harris et al., 2002). PGE2 is the most abundant prostanoid in humans and involved in regulating many different biological functions ranging from reproduction to neuronal, metabolic and immune functions (Dey et al., 2006; Legler et al., 2010; Park et al., 2006; Wang et al., 2007). Synthesized PGE2 is actively transported through the membrane by the ATP-dependent multidrug resistance protein-4 (MRP4) or diffuses across the plasma membrane (Park et al., 2006) PGE2 then acts locally through binding of EPs (Sugimoto and Narumiya, 2007). PGE2 is not stored but rapidly metabolized within minutes by cytosolic enzymes (Tai et al., 2006).

3. Biological roles of PGE2

PGE2 has been described as a regulator of numerous physiological functions. One of the most important features of PGE2 is its vasodilatory activity including modulation of hemodynamics in the kidney (Fortier et al., 2008), contraction and relaxation of smooth muscle cells associated with blood pressure control (Hristovska et al., 2007). In inflammation, PGE2 is of particular interest because it is involved in all processes leading to the classic signs of inflammation (Funk, 2001; Harris et al., 2002). PGE2 has been referred to as a classical pro-inflammatory mediator. However, the role of PGE2 in the regulation of inflammatory responses is even more complex. PGE2 also exerts anti-inflammatory responses (Sugimoto and Narumiya, 2007).

Since PGE2 can be produced by virtually any cells of the human body, either constitutively or upon stimulation, and signals through different receptors, its biological effects are diverse and of an astounding complexity, depending on the amount of PGE2 available within the microenvironment of diverse tissues and on four EPs (Sugimoto and Narumiya, 2007).

EP1 couples to Gαq protein and signals through the phospholipase C (PLC)/inositol-1,4,5-trisphosphate (IP3) pathway resulting in the formation of the second messengers diacylglycerol (DAG) and IP3, with the latter rapidly liberating calcium ions from intracellular stores (Legler et al., 2010). EP2 and EP4 receptor subtypes couple to Gαs and its activation leads to increased cyclic adenosine monophosphate (cAMP) production (Legler et al., 2010). In contrast, EP3 couples to Gαi for signaling and inhibits adenylyl cyclase (AC) activation resulting in decreased cAMP concentrations (Table 1).

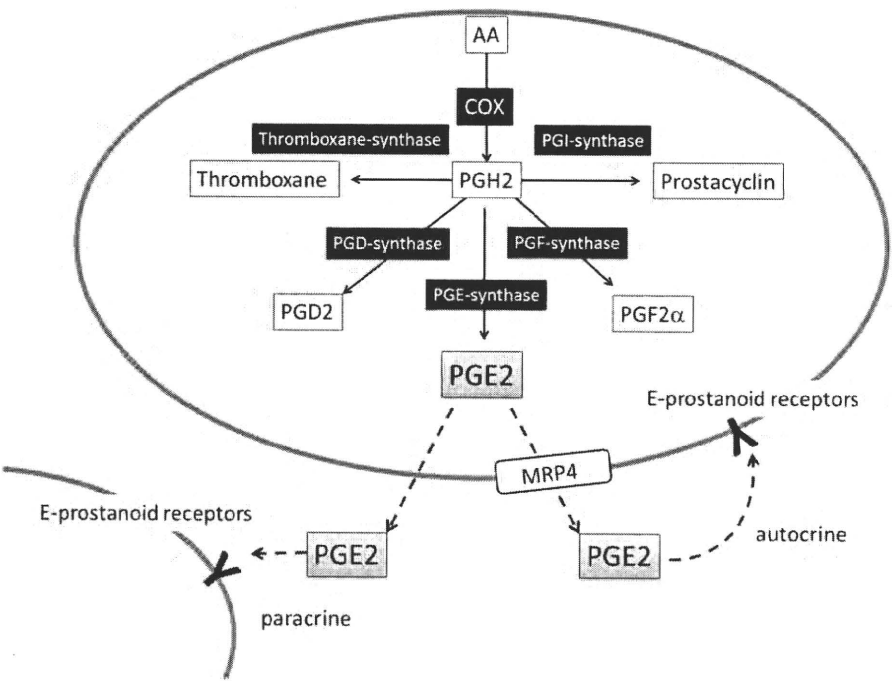

Fig. 1. PGE2 biosynthesis and metabolism. AA: arachidonic acid, COX: cyclooxygenase, PGH2: prostaglandin H2, PGD2: prostaglandin D2, PGF2α: prostaglandin F2α, PGE2: prostaglandin E2, PGI: prostaglandin I2, MRP4: multidrug resistance protein-4.

Table 1
E-prostanoid receptors; signaling, second messenger, effect on vasculature, effect on neurons and possible actions in cochleae. EP1: E-prostanoid receptor 1, EP2: E-prostanoid receptor 2, EP3: E-prostanoid receptor 3, EP4: E-prostanoid receptor 4, PLC: phospholipase C, IP3: inositol-1,4,5-trisphosphate, AC: adenylyl cyclase, cAMP: cyclic adenosine monophosphate.

	Signaling	Second messenger	Vasculature	Neuron	Possible actions in cochleae
EP1	PLC/IP3	Increase IP3, Ca^{2+}	Vasoconstriction	Toxic	Decrease blood flow Ototoxic
EP2	AC/cAMP	Increase cAMP	Vasodilation	Protective in acute injury Toxic in chronic inflammation	Increase blood flow Protective
EP3	AC/cAMP	Decrease cAMP	Vasoconstriction	Toxic	Decrease blood flow Ototoxic
EP4	AC/cAMP	Increase cAMP	Vasodilation	Protective	Increase blood flow Protective

Interestingly, biological actions of some EPs are contradictory to those of other EPs. In hemodynamics, EP1 induces vasoconstriction in the peripheral vasculature, while EP2 and EP4 signaling induces vasodilation. The activation of EP2 and EP4 increases intracellular cAMP levels, while EP3, which is regarded as an inhibitory receptor, decreases cAMP formation. Recent studies with gene targeted mice, in which single EP was deleted, gave new insights on the various actions of PGE2 (Sugimoto and Narumiya, 2007), although their auditory function has not been examined in detail. Therefore, the development of subtype-specific EP agonists/antagonists will boost pharmacological actions of PGE1 or PGE2.

4. EP signaling in the central nervous system

In the central nervous system, some subtype-specific EP signaling events mediate toxic effects but others appear to mediate paradoxically protective effects. In addition, protective or toxic effects of a particular EP signaling pathway can differ depending on the context of cerebral injury, for example, in excitotoxicity/hypoxia paradigms versus inflammatory-mediated secondary neurotoxicity (Andreasson, 2010).

Regarding pathological roles, in general, EP1 signaling plays neurotoxic roles. In N-methyl-D-asparate (NMDA) excitotoxicity, increased calcium ion influx through NMDA receptors is a primary mechanism for neurotoxicity. The activation of EP1 causes an increase of intracellular calcium ion levels, which enhances NMDA neurotoxicity. Actually, the normalization of intracellular calcium ion levels with EP1 blockade or genetic deletion attenuates NMDA neurotoxicity (Ahmad et al., 2006; Kawano et al., 2006). EP1 is also known to induce vasoconstriction in the peripheral vasculature, indicating that EP1 signaling is involved in ischemia-induced brain injury (Saleem et al., 2007). In addition, direct effects of EP1-mediated neurotoxicity have been demonstrated in models of neurodegenerative disease including Parkinson's diseases (Carrasco et al., 2007).

In contrast to EP1, EP2 signaling plays a role in neuroprotection against acute neurotoxic events. Direct actions of the EP2 in neurons mediate pro-survival and anti-apoptotic functions (Jiang et al., 2010). Activation of the EP2 is neuroprotective in paradigms of NMDA toxicity, which is mediated by protein kinase A (PKA) activation via cAMP signaling (Liu et al., 2005; McCullough et al., 2004; Mori et al., 2009). However, stimulation with PGE2 has no effect on NMDA toxicity, which is consistent with the idea of toxic EP1 activation and protective EP2 activities. In setting of cerebral ischemia, EP1 activation decreases blood flow, whereas EP2 activation increases blood flow by vasodilation. Further complexity is emerging in EP2 effects in the central nervous system. Effects of the EP2 activation differ depending on the context of brain injury. In acute toxicity, EP2 signaling is neuroprotective, while in chronic inflammation EP2 signaling shows pro-inflammatory neurotoxic effects. In lipopolysaccharide (LPS)-mediated toxicity, microglial EP2 activation induces generation of reactive oxygen species (ROS) (Montine et al., 2002). In short, neuronal EP2 signaling mediates neuroprotection in acute injury models via cAMP-PKA signaling, while microglial EP2 signaling leads to secondary neurotoxicity via ROS generation.

The EP4 receptor is positively coupled to cAMP production similarly to the EP2 receptor. EP4 signaling functions to confer neuroprotection in excitotoxic (Ahmad et al., 2005) and ischemia (Li et al., 2008) models, likely EP2 signaling. The EP4 receptor has also been demonstrated to modulate the inflammatory response. As described above, EP2 signaling is neurotoxic in models of chronic inflammation, while EP4 signaling mediates an anti-inflammatory effect in some contexts. EP4 signaling suppresses brain inflammation in models of LPS-induced inflammation (Shi et al., 2010) and multiple sclerosis (Esaki et al., 2010). Pharmacological inhibition of EP4 signaling reverses attenuation of spinal cord injury by PGE1 analog (Umemura et al., 2010).

The EP3 receptor is regarded as an inhibitory receptor, because of its effect decreasing cAMP production. Hence, EP3 signaling may be neurotoxic by its inhibitory effects against EP2 and/or EP4 neuroprotective effects. In fact, an EP3 antagonist attenuates and an EP3 agonist augments glutamate-induced excitotoxicity in hypocampal neurons (Ikeda-Matsuo et al., 2010). In addition, local application of EP3 agonist worsens infarct volume in a model of transient focal ischemia (Ahmad et al., 2007). However, in models of neurological diseases, the function of the EP3 receptor so far is not firmly defined. The murine EP3 receptor consists of three distinct isoforms derived by alternative splicing of the carboxy terminus; these isoforms differ in downstream signaling pathways, desensitization, and constitutive activity (Bilson et al., 2004; Hasegawa et al., 2000). Thus, genetic deletion of EP3 results in total ablation of all three isoforms whereas administration of EP3 agonist may activate one or more isoforms depending on the cellular expression patterns of the EP3 isoforms.

These findings indicate that the development of subtype-specific EP agonists or antagonists is necessary for establishment of novel therapeutic approaches for neuronal degeneration associated with COX-PGE2 signaling, and emphasize importance of selective stimulation or inhibition of specific EP signaling depending on contexts of neuronal diseases.

5. COX-PGE2 signaling in cochleae

PGs are generated by oxidation of AAs by cyclooxygenase-1 (COX-1) and/or -2 (COX-2) and act in an autocrine and paracrine fashion. The presence of COX-1 and COX-2 in the cochlea was demonstrated using immunohistochemistry. The constitutive expression of COX-1 was found in various types of cochlear cells, while COX-2 expression in normal cochleae is still controversial (Stjernschantz et al., 2004; Ziegler et al., 2004). Immunoreaction for COX-1 was found in the stria vascularis, spiral ligament, spiral limbus, spiral ganglion and organ of Corti. These findings indicate the constitutive formation of PGs in the cochlea. In vitro radioimmunoassay using cochlear tissue supports this hypothesis of constitutive PG formation in cochleae, which showed the synthesis of PGD2, PGF2α and most abundantly PGE2 in cochleae (Kawata

et al., 1988). Altogether, PGs, most abundantly PGE2, is constitutively generated in the cochlea.

In models of noise-induced hearing loss as a pathophysiological condition of cochleae, alterations in expression of COX-1 and COX-2 and effects of several COX inhibitors were investigated. Alterations in COX-1 or COX-2 expression were examined using guinea pig exposed to 8 kHz tone bursts at 70 or 90 dB sound pressure level (SPL) for 60 min, which is moderate intensity and induces limited irreversible degeneration in cochleae (Heinrich et al., 2006, 2010). Both COX-1 and COX-2 expression in the organ of Corti decreased after noise exposure. A slight increase of COX-1 expression was found only in the nerve fibers of the osseous spiral lamina. No remarkable alteration in COX-2 expression was found in other cochlear constructions. These findings indicate that noise-induced damage causes a decrease of COX enzymes in the organ of Corti, and no obvious up-regulation of COX enzymes in cochleae. Effects of several COX inhibitors were tested in mice exposed to an intense noise, a 4 kHz pure tone of 128 dB SPL for 240 min (Hoshino et al., 2008). Indomethacine, a COX-1 inhibitor, showed significant hair cell protection in the apical portion of cochleae and attenuation of noise-induced hearing loss at low frequencies. Three COX-2 inhibitors exhibited no protective effects against noise trauma. Altogether, excessive formation of PGs including PGE2 may not occur in cochleae after noise exposure.

6. Expression of EPs in cochleae

Biological effects of PGE2 on cochleae may largely depend on the distributions and amounts of EPs in cochleae. Previously, the expression of EP1 and EP3 was investigated in normal guinea pig cochleae using immunohistochemistry (Stjernschantz et al., 2004). EP1 expression was found in the stria vascularis, spiral ligament, spiral ganglion, and organ of Corti. The expression of EP3 exhibited a similar distribution to that of EP1. Recently, the expression of EP2 and EP4 in mouse cochleae was examined by immunohistochemistry and reverse transcription polymerase chain reaction (Hori et al., 2009, 2010). The expression of EP2 and EP4 was found in the stria vascularis, spiral ligament, spiral ganglion and organ of Corti similarly to that of EP1 or EP3. These findings showed the presence of four subtypes of PGE2 receptors, EP1–4, in cochlear structures that play important roles in auditory function. Hence, activation or inhibition of specific EP signaling is important to boost therapeutic potential of PGE2 signaling in cochleae, probably depending on contexts of cochlear diseases similarly to the central nervous system.

7. Possible role of EP signaling in the cochlear blood flow

PGE1 has long been used for the treatment of SSHL aiming to increase cochlear blood flow (Nakashima et al., 2003). Expression of EP1–4 in the stria vascularis of cochleae suggests an involvement of EP signaling in the regulation of the cochlear blood flow. In general, EP2 and EP4 mediate vasodilatation, and EP1 and EP3 mediate vasoconstriction in many tissues (Legler et al., 2010). Animal experiments demonstrated an increase of cochlear blood by local application of PGE1 or PGE2 using a laser Doppler flowmeter (Umemura and Nakashima, 1997; Rhee et al., 1999; Tominaga et al., 2006), which indicates that EP2 and/or EP4 signaling dominantly works in the cochlear vasculatures rather than EP1 or EP3. In addition to direct actions of EP2 and/or EP4 activation, vasodilatation mediated by vascular endothelial growth factor (VEGF) could be involved in mechanisms for vasodilatation in cochleae by PGE2 signaling. Activation of EP2 and EP4 signaling in cochleae induces the formation of VEGF, which has effects of vasodilatation (Huang et al., 2010), in cochleae (Hori et al., 2010).

On the other hand, noise trauma is known to reduce cochlear blood flow (Miller et al., 2003; Yamane et al., 1991). Studies on expression of COX enzymes in cochleae indicated down-regulation of COX enzymes following noise exposure (Heinrich et al., 2006, 2010), which can lead to a decrease of endogenous PGE2 levels in cochleae. Therefore, down-regulation of PGE2 signaling, particularly EP2 and EP4 signaling, could be involved in mechanisms for reduction of cochlear blood flow due to noise exposure.

However, the localization of EPs in the stria vascularis was demonstrated in a light microscopic level. It is, therefore, unclear which type of cells in the stria vascularis, marginal, intermediate or basal cells, express which subtype of EPs. In addition, alteration in expression of EPs in the stria vascularis following noise exposure has not been investigated. Further studies are required to determine distinct roles of EP signaling in the regulation of cochlear blood flow.

8. Possible role of EP signaling in the cochlear sensory system

Previous studies have demonstrated an involvement of excitotoxicity in cochlear degeneration due to noise trauma (Duan et al., 2000; Hakuba et al., 2000; Puel et al., 1998). Excessive efflux of glutamate from inner hair cells causes degeneration in afferent nerve endings of spiral ganglion neurons following noise exposure. In the central nervous system, activation of EP2 or EP4 functions to confer neuroprotection (Ahmad et al., 2005; McCullough et al., 2004; Liu et al., 2005), while EP1 or EP3 signaling plays neurotoxic roles in models for excitotoxicity (Candelario-Jalil et al., 2005; Ahmad et al., 2006; Ikeda-Matsuo et al., 2010). Therefore, activation of EP2 or EP4 or inhibition of EP1 or EP3 was expected to protect cochlear sensory cells against noise trauma. Cochlear protection by local application of an EP4 agonist, ONO-AE1-329 (Ono Pharmaceutical, Co., Ltd., Osaka, Japan), was investigated using a guinea pig model of noise-induced hearing loss (Hori et al., 2009). In histology, we focused on cochlear outer hair cells, because they are susceptible for noise trauma. Local application of an EP4 agonist before or after noise exposure successfully attenuates noise-induced hearing loss and protects outer hair cells from noise trauma. Therefore, specific activation of EP4 signaling in cochleae may be an effective strategy for attenuation of cochlear damage associated with excitotoxicity. Since ischemic injury of cochleae also involves excitotoxicity (Hakuba et al., 1997; Pujol et al., 1992), specific activation of EP4 signaling might have protective effects on cochlear sensory cells against ischemia-induced damage similarly to the central nervous system (Li et al., 2008).

Activation of EP2 or EP4 can directly promote the survival of cochlear sensory cells via cAMP-mediated signaling pathways. EP2 and EP4 signaling is also known to act as neuroprotective via induction of growth factor formation (Chang et al., 2005; Takahashi et al., 1996). Since numerous studies demonstrated formation of VEGF by activation of EP2 or EP4 signaling in other systems (Chang et al., 2005; Inoue et al., 2002; Weiss et al., 2004; Bradbury et al., 2005; Jain et al., 2008), we examined VEGF formation in inner ears following local application of an EP2, ONO-AE1-259-01 or EP4 agonist, ONO-AE1-329 (both from Ono Pharmaceutical, Co., Ltd.), and the localization of VEGF and VEGF receptors in mouse cochleae (Hori et al., 2010). The levels of VEGF mRNA and protein increased after local EP2 or EP4 agonist application, showing VEGF formation in inner ears in response to EP2 or EP4 activation. Immunohistochemistry for VEGF indicates that VEGF is predominantly generated in the spiral ganglion neurons (Hori et al., 2010). The expression of VEGF receptors was found in the stria vascularis, spiral ligament, spiral ganglion and organ of

Corti. Previous studies have indicated that VEGF has protective effects on cochlear hair cells (Picciotti et al., 2006; Selivanova et al., 2007). Therefore, VEGF formation via EP4 activation may contribute to protection of outer hair cells against noise trauma by local EP4 agonist treatment.

9. Possible role of EP signaling in the cochlear immunity

PGE2 has been referred to as a classical pro-inflammatory mediator. The relevance of PGs during the promotion of inflammation is emphasized by the effectiveness of non-steroidal anti-inflammatory drugs (NSAIDs) acting as COX inhibitors (Simmons et al., 2004). However, the role of PGE2 in the regulation of immune responses is even more complex. Studies on knock-out mice deficient for individual EPs clearly revealed that PGE2 not only acts as a pro-inflammatory mediator, but also exerts anti-inflammatory responses (Sugimoto and Narumiya, 2007). In addition, recent investigations have demonstrated significant roles of PGE2 signaling, in particular EP2 and EP4 signaling, in innate and adaptive immune systems (Divangahi et al., 2010; Gagliardi et al., 2010; Mandapathil et al., 2010; Zhu et al., 2010). PGE2 is classically known as an immunosuppressant, because it inhibits helper T cell differentiation (Harris et al., 2002; Sugimoto and Narumiya, 2007). However, recent studies demonstrate that PGE2 can function as an immunoactivator via EP2 and EP4 under some conditions (Krause et al., 2009; Yao et al., 2009).

The innate immune system of the central nervous system is principally composed of microglia and astrocytes (Rothwell and Luheshi, 2000). Microglia play a key role in EP2-mediated neurotoxic effects in models of chronic inflammation (Jin et al., 2007; Liang et al., 2008; Montine et al., 2002). Activation of EP2 signaling in microglia induces ROS generation, which results in degeneration of neurons. Expression of the EP2 receptor is highly inducible in microglia of the cerebral cortex and hippocampus in the LPS model of innate immunity (Zhang and Rivest, 1999). Interestingly, EP4 signaling mediates opposite actions of EP2 signaling in the context of chronic inflammation in the central nervous system, although the EP4 receptor is positively coupled to cAMP production similarly to the EP2 receptor. EP4 signaling mediates an anti-inflammatory effect in models of chronic inflammation. EP4 signaling suppresses brain inflammation in models of LPS-induced inflammation (Shi et al., 2010) and multiple sclerosis (Esaki et al., 2010).

The immune system of the inner ear has not been fully understood. However, recent studies indicate importance of the spiral ligament in the immune reaction in the cochlea. Macrophages and/or microglia are constitutively present in the spiral ligament and accumulate in response to injury (Hirose et al., 2005; Okano et al., 2008; Sato et al., 2008). The spiral ligament fibrocytes produce cytokines in response to exposure to bacterial toxins (Moon et al., 2006, 2007) and noise exposure (Fujioka et al., 2006). EP signaling could mediate immune reactions in the spiral ligament, because of the presence of EPs in the spiral ligament (Hori et al., 2009, 2010; Stjernschantz et al., 2004).

However, roles of EP signaling in the spiral ligament and in models of chronic inflammation in cochleae have not been investigated. In future, responses of spiral ligament fibrocytes and cochlear microglia/macrophages for individual EP signalings should be examined to reveal roles of EP signaling in the regulation of immune responses in cochleae. Roles of EP2 signaling in models of cochlear chronic inflammation are particularly interesting, because EP2 activation confers neuroprotection in acute injury models, but mediates neurotoxicity in chronic inflammation models in the central nervous system.

10. Conclusions

Accumulating evidence for EPs indicate significant roles of individual EP signaling in neurodegenerative diseases. In the inner ear, PGs were paid particular attention as regulators of cochlear blood flow. In addition, PGE1 has long been used as a vasodilator for the treatment of SSHL in clinic. However, little is known about roles of EP signaling in the auditory function. Previous studies have demonstrated that PGE2 is constitutively produced in the cochlea and all EP subtypes are expressed in various types of cochlear cells, suggesting that EP signaling may play roles in physiology and pathophysiology of the cochlea. It is necessary to examine the auditory function in individual EP-deficient mice in normal and pathological conditions, which may bring new insights on roles of PGE2 signaling in cochleae. In addition, various antagonists or agonists for individual EPs are available, which may be useful tools for elucidation of roles of EP signaling in the auditory function. We believe that future investigations on EP signaling in cochleae must lead to the development of novel therapeutic options for SNHL.

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The Laryngoscope

Adipose Tissue-Derived Stromal Cells Protect Hair Cells From Aminoglycoside

AQ1 Atsuhiro Yoshida[a]; Shin-Ichiro Kitajiri[a]; Takayuki Nakagawa[a]; Kento Hashido[a]; Takatoshi Inaoka[a]; Juichi Ito[a]

Background: Previous studies have demonstrated the therapeutic paracrine activity of adipose tissue-derived stromal cells (ADSCs). This study aimed to examine the ADSC potential for protecting auditory hair cells from aminoglycoside toxicity via paracrine of multiple growth factors and cytokines.

Study Design: Experimental study.

Methods: We assessed hair cell protection from neomycin toxicity by ADSC-derived factors using an explant culture system, in which cochlear explants and ADSCs were separated by a culture mesh insert to avoid direct contact. We measured the levels of growth factors and cytokines in ADSC culture media using an enzyme-linked immunosorbent assay (ELISA).

Results: Neomycin induced severe degeneration of auditory hair cells in cochlear explants, but co-culture with ADSCs significantly increased the number of surviving hair cells in explants. ELISA analysis revealed that ADSCs secreted insulin-like growth factor-1, nerve growth factor, vascular epithelial growth factor, transforming growth factor β1, monocyte chemotactic protein-1, and most prominently hepatocyte growth factor.

Conclusions: These findings demonstrate that ADSCs have the capacity to protect auditory hair cells, and can be a useful strategy to develop therapy for deafness in the clinic. The multiple paracrine growth factors and cytokines secreted by ADSCs might be involved in this effect.

Key Words: Adipose tissue-derived stromal cell, protection, cochlea, cell therapy.

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INTRODUCTION

Sensorineural hearing loss (SNHL) is one of the most common disabilities, occurring in 60% of individuals aged over 65 years[1] and in 1/1,000-2,000 newborns.[2] However, therapeutic options for SNHL are limited to hearing aids and cochlear implants for chronic SNHL. For acute SNHL, the most acceptable therapeutic option is systemic glucocorticoid application, but approximately 30% of patients exhibit no response following treatment.[3] In addition, there are currently no secondary choices of therapeutic options that have demonstrated clinical efficacy. A major obstacle for the development of novel therapeutic options for SNHL is the inability to regenerate sensory hair cells in the mammalian cochleae.[4] Therefore, practical strategies have focused on the protection of cochlear hair cells.

Previous experimental studies have demonstrated the efficacy of several growth or neurotrophic factors in the protection of cochlear hair cells against various ototoxic insults. A combination of glial cell line-derived neurotrophic factor and transforming growth factor β1 (TGF-β1) reduces the loss of hair cells due to aminoglycoside ototoxicity.[5] Local application of insulin-like growth factor-1 (IGF-1) into cochleae provides functional and histological protection of cochlear hair cells against noise trauma[6,7] and ischemic injury.[8] More recently, the protective effects of hepatocyte growth factor (HGF) on cochlear hair cells against aminoglycoside toxicity[9] and noise trauma[10] have also been reported.

Transplantation of stem or stem cell-like cells is an alternative strategy for the local, sustained delivery of growth or trophic factors and cytokines into tissues, because they have the capacity to secrete several growth factors and cytokines. For this purpose, stromal cells derived from adipose tissue have been frequently used. Adipose tissue-derived stromal cells (ADSCs) are known to secrete multiple growth factors and cytokines,[11] and ADSC transplantation has contributed to angiogenesis[12,13] or functional restoration of the liver[11] via paracrine of solvable factors. We therefore highlighted the potential of ADSCs for paracrine of multiple growth factors and cytokines, and investigated the effects of ADSC on the protection of cochlear hair cells in an explant culture system where cochlea explants and ADSCs were co-cultured without their direct contact. Here, we report on the significant attenuation of aminoglycoside-induced damage in cochlear hair cells co-cultured with ADSCs.

From the Department of Otolaryngology–Head and Neck Surgery (A.Y., S.-I.K., T.N., K.H., T.I., J.I.), Graduate School of Medicine, Kyoto University, Kyoto; and the Therapeutic Technology R&D Department (K.H.), Medical Technology R&D Division, Olympus Corporation, Tokyo, Japan.

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Send correspondence to Takayuki Nakagawa, Department of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyoku, Kyoto 606-8507, Japan. E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

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MATERIALS AND METHODS

Animals

Imprinting control region (ICR) mice (Japan SLC, Hamamatsu, Japan) used in this study were cared for in the Institute of Laboratory Animals of the Kyoto University Graduate School of Medicine, Japan. The Animal Research Committee of the Kyoto University Graduate School of Medicine approved all experimental protocols, which were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Isolation and Expansion of ADSCs

Mouse ADSCs were isolated and expanded according to methods described previously.[14] Briefly, femoral adipose tissue samples were obtained from 20 male ICR mice at 8 weeks of age under general anesthesia with midazolam (10 mg/kg; Astellas, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Tokyo, Japan). Samples obtained from five animals were transferred into one conical tube. A total of four conical tubes from 20 mice were used to isolate ADSCs.

Adipose tissues were digested in a lacto-Ringer solution containing 0.035 mg/mL Liberase Blendzyme 3 (Roche Applied Science, Mannheim, Germany) under agitation for 40 minutes at 37°C, then rested for 5 minutes to separate the stromal cell fraction from adipocytes. The stromal cell fraction was filtered through a 100 μm cell strainer (BD Biosciences, San Jose, CA) and centrifuged at 500 $\times$ g for 5 minutes. The cell pellet was resuspended in a lacto-Ringer solution and filtered through a 40 μm cell strainer (BD Biosciences). Isolated cells were seeded at 6×10^4 cells/cm^2 in 10-cm plastic plates and allowed to adhere in basic medium (DMEM/F12 with 10% fetal bovine serum, 50 μg/mL gentamycin, 250 ng/mL amphotericin B) for 24 hours. The medium was changed once daily until the cells were 80% confluent. Nonadherent cells were removed during the medium-change procedure. After the fourth passage, the cells were seeded at 1.6×10^4 cells/mL in 6-well plastic plates and cultured until they were 80% confluent. Culture media were then replaced with DMEM/F12 without supplements. ADSCs were cultured for a further 7 days without medium changes.

Cochlear Explants

Postnatal day 3, ICR mice were deeply anesthetized with sevoflurane and decapitated. The temporal bones were dissected, and the cochleae freed from the surrounding tissue and placed in 0.01-M phosphate buffered saline (pH 7.4). After removing the cochlear lateral wall, the cochlear epithelia were dissected from the cochlear modiolus. The tissue samples were then placed on culture mesh inserts (Falcon, Billerica, MA) and cultured initially in serum-free minimum essential medium (MEM) (Invitrogen, Carlsbad, CA), supplemented with 3 g/L glucose (Wako Pure Chemicals, Osaka, Japan) and 0.3 g/L penicillin G (Wako Pure Chemicals), for 24 hours at 37°C in humidified (95%) air at 5% atmospheric CO_2. In total, 26 cochlear explants were used. As the hair cells in the apex are resistant to aminoglycosides, the basal (60%–80% from the apex) and upper-basal portions (40%–60% from the apex) of the cochlea were used in this study. We used five cochlea explants without any toxic drugs to confirm that this explant culture system did not damage the hair cells.

Neomycin Application and Co-Culture of Cochlear Explants With ADSCs

Neomycin (Wako Pure Chemicals) was added to the culture wells of 6-well plastic plates containing ADSCs at a final concentration of 1 mM. To avoid attachment between ADSCs and cochlear explants, the latter (n = 9) were transferred with culture-mesh inserts to culture wells containing ADSCs and 1 mM neomycin. In this co-culture study, we used ADSCs from the same origin. Other cochlear explants (n = 12) were transferred with culture-mesh inserts to culture wells containing fresh DMEM/F12 supplemented with 1 mM neomycin, and served as controls. Cultures were maintained for 24 hours, then fixed for 15 minutes in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

Hair Cell Protection Assay

Cochlear explants were provided for the immunostaining of myosin VIIa and F-actin staining with phalloidin. Specimens were incubated with primary rabbit polyclonal antibodies against myosin VIIa (1:500; Proteus Bioscience Inc., Ramona, CA). Alexa-Fluor 568 goat anti-rabbit IgG (1:500; Invitrogen) was used as the secondary antibody. Specimens were then incubated in fluorescein isothiocyanate-conjugated phalloidin (1:80; Invitrogen) and viewed with a Leica TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany).

To quantify cochlear hair cell damage, inner hair cells (IHCs) and outer hair cells (OHCs) with organized stereocilia were counted over 200-μm-longitudinal distances from two separate regions in the basal and upper-basal portions, respectively. The average value was used as the data for each culture. All values are expressed as the mean $\pm$ 1 standard error (SE). Differences in the number of hair cells with organized stereocilia between specimens co-cultured with ADSCs and control specimens cultured without ADSCs were analyzed by unpaired t tests. P values $<.05$ were considered to be statistically significant.

Measurement of Growth Factors and Cytokines in Culture Media

We expanded ADSCs from the four conical tubes into four separate culture wells. Following a 7-day culture of ADSCs, 500 μL of culture media were collected from each culture well, and used for an enzyme-linked immunosorbent assay (ELISA) of growth factors and cytokines using the assay kits shown in Table I. ELISA analyses were performed according to the manufacturers' instructions. Triplicate measurements were performed for each sample, and an average taken. All values are expressed as the mean $\pm$ 1 SE. T1

RESULTS

Hair Cell Protection by ADSCs

We estimated the protective effects of ADSC on cochlear hair cells against neomycin toxicity using an explant culture system. Previously, we established an explant culture model to analyze aminoglycoside toxicity on cochlear hair cells, in which we confirmed that any damage was not seen in the hair cells without toxic drug treatments, and a 24-hour culture with 1 mM neomycin destroyed approximately 70% of IHCs and OHCs in the explants.[9] These were deemed suitable conditions to evaluate the protective effects on cochlear hair cells. Morphology of the hair cell surface in cochlear explants is shown in Figure 1. In control specimens that were cultured with 1 mM neomycin alone, both IHCs and OHCs were severely degenerated in the basal (Fig. 1d–1f) and upper-basal portions (Fig. 1j–1l), as observed in our previous investigation.[9] In the basal portion of cochlear F1

TABLE I.
Measured Growth Factors, Cytokines, and Enzyme-Linked Immunosorbent Assay Kits.

Protein	Kit Name	Supplier
BDNF	Human BDNF Quantikine kit	R&D systems, Minneapolis, MN
BMP2	Human BMP2 Quantikine kit	R&D systems, Minneapolis, MN
EGF	EGF Mouse, ELISA Kit, Quantikine	R&D systems, Minneapolis, MN
bFGF	Human FGFb Quantikine kit	R&D systems, Minneapolis, MN
HGF	HGF, DuoSet(R)ELISA Development kit	R&D systems, Minneapolis, MN
IGF-1	Mouse IGF-1 Quantikine ELISA Kit	R&D systems, Minneapolis, MN
NGF	NGF Emax (R) ImmunoAssay System	Promega, Madison, WI
PDGF	Mouse/Rat PDGF-BB Quantikine ELISA Kit	R&D systems, Minneapolis, MN
TGF-b1	Canine, Mouse, Rat, Porcins TGF-b1 Quantikine kit	BenderMedSystems, Vienna, Austria
VEGF	VEGF, Mouse, ELISA Kit, RayBio	BenderMedSystems, Vienna, Austria
G-CSF	Mouse G-CSF Single Analyte ELISArray Kit	SABiosciences, Frederick, MD
GM-CSF	Mouse GM-CSF Platinum ELISA	BenderMedSystems, Vienna, Austria
M-CSF	M-CSF ELISA	Peprotech, Rocky Hill, NJ
MCP-1	Mouse MCP-1 kit	BenderMedSystems, Vienna, Austria
MIP-1a.	MIP-1α(Ccl3)ELISA Kit, Single Analyte ELISArray	Peprotech, Rocky Hill, NJ
MIP-1b	MIP-1β(Ccl4) ELISA Kit, Single Analyte ELISArray	Peprotech, Rocky Hill, NJ
SDF-1	Mouse SDF-1, Quantikine kit	R&D systems, Minneapolis, MN

BDNF: Brain-derived nuerotrophich factor, BMP2: Bone morphogenetic protein-2, EGF: Epidermal growth factor, bFGF: basic fibroblast growth factor, HGF: Hepatocyto growth factor, IGF-1: Insulin-like growth factor-1, NGF: Nerve growth factor, PDGF: Platelet-derived growth factor, TGF-b1: Transforming growth factor-beta1, VEGF: Vascular endothelial growth factor, G-CSF: Granulocyte-colony stimulating factor. GM-CSF: Granulocyte-macrophage colony-stimulating factor, M-CSF: Macrophage colony-stimulating factor, MCP-1: Monocyte chemotactic protein-1MIP-1a: Macrophage inflammatory protein-1 alpha, MIP-1b: Macrophage inflammatory protein-1beta, SDF-1a Stromal cell-derived factor-1alpha.

explants, 4.7 ± 0.5 IHCs and 12.4 ± 1.5 OHCs were present in a 200-μm length region, and the numbers of surviving IHCs and OHCs were 4.8 ± 0.9 and 12.1 ±1.9 in a 200-μm length region of the upper-basal portion of cochlear explants, respectively.

In contrast to control specimens, IHCs and OHCs were well maintained in both the basal (Fig. 1a–1c) and upper-basal portions (Fig. 1g–1i) of cochlear explants following co-culture with ADSCs. The numbers of surviving IHCs and OHCs in the basal portion were 20.3 ± 1.1 and 61.1 ± 4.6 in a 200-μm length region, and those in the upper-basal portion were 19.0 ± 0.7 and 64.4 ± 4.2. Differences in numbers of IHCs and OHCs between co-cultured and control specimens were

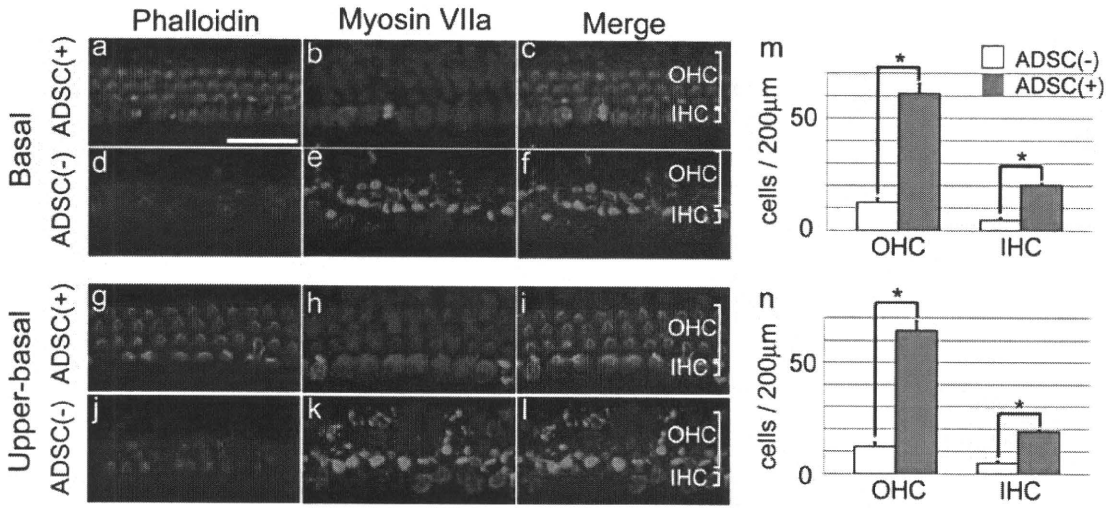

Fig. 1. Hair cell protection by paracrine effects of adipose tissue-derived stromal cells (ADSCs) against aminoglycoside toxicity. Basal (a–f) and upper-basal portions (g–l) of cochlear epithelia cultured with neomycin are shown. Phalloidin staining and immunostaining for myosin VIIa demonstrate that inner hair cells (IHCs) and outer hair cells (OHCs) are well maintained in cochleae cultured with ADSCs (a–c, g–i), whereas severe degeneration is found in both IHCs and OHCs in cochleae cultured without ADSCs (d–f, j–l). Quantitative analyses reveal significant differences in surviving IHC and OHC numbers between the two groups in the basal (m) and upper-basal (n) portions, respectively (*). Scale bar in (a) represents 50 μm (a–l).

COLOR

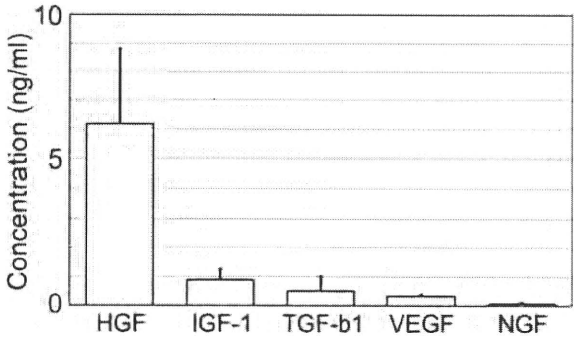

Fig. 2. Paracrine factors in ADSC-conditioned media. Hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), and nerve growth factor (NGF) were detected by enzyme-linked immunosorbent assay (ELISA). Bars represent 1 standard error.

statistically significant ($P < 0.01$ for each condition) (Fig. 1m–1n), demonstrating that co-culture with ADSCs significantly attenuates neomycin-induced damage of cochlear hair cells. This suggests that ADSCs may secrete protective factors for cochlear hair cells into culture media.

Growth Factors and Cytokines Secreted by ADSCs

To examine the ability of ADSCs to secrete growth or trophic factors and cytokines, we measured the protein levels of several factors in culture media collected after a 7-day culture of ADSCs using ELISA. Analyses revealed the presence of five growth factors: HGF, IGF-1, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and TGF-β1 at detectable levels for each assay kit (Fig. 2). The protein levels of brain-derived neurotrophic factor, bone morphogenic protein-2, epidermal growth factor, basic fibroblast growth factor, and platelet-derived growth factor were undetectable by ELISA, whereas the protein level of HGF was 6154.62 ± 2673.18 pg/mL. The levels of IGF-1, TGF-β1, VEGF, and NGF were 889.69 ± 355.21, 502.00 ± 502.00, 327.53 ± 63.90, and 76.53 ± 33.89 pg/mL, respectively. For cytokines, ELISA analyses revealed the presence of one cytokine: monocyte chemotactic protein-1 (MCP-1), whereas the protein levels of other cytokines, granulocyte-colony stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, macrophage inflammatory protein-1α, β (MIP-1α, β), and stromal cell-derived factor-1α (SDF-1α), were not detectable. The level of MCP-1 was 6310.67 ± 2101.91 pg/mL. These findings demonstrate that HGF, IGF-1, VEGF, NGF, TGF-β1 and MCP-1 are secreted from ADSCs.

DISCUSSION

Cell transplantation can contribute to the functional restoration of various organs through two different mechanisms. One is the differentiation of transplants into functional cells and the formation of functional connections with host cells. The second is paracrine of therapeutic factors from transplanted cells that promote self-repair systems in host organs. Several cell types have had their potential tested for use as transplants in cell-based therapy. Of these, mesenchymal stromal cells have often been used because of their ready and easy availability from bone marrow or adipose tissue, particularly in the case of autologous cells.[15] Recent studies have also demonstrated the high potential of human ADSCs for the secretion of various growth factors.[11] Furthermore, ADSCs have some advantages over bone marrow-derived stromal cells (BMSCs). First, collecting ADSCs is easier and less invasive than collecting BMSCs. Complications of bone marrow aspiration include osteomyelitis and accidental puncture of adjacent vital organs. Second, we can collect larger amount of cells at one time from adipose tissue than from bone marrow. It is possible that multiple passages of ADSCs are not needed before there are enough cells to be transplanted. Third, autologous fat transplantation have been performed for improving facial and body contour depressions and scars since 1898,[16] and the safety of adipose tissue transplantation is well established. For such reasons, we investigated the paracrine effects of ADSCs in the protection of mammalian cochlear hair cells.

In this study, we tested the potential of ADSCs in the rescue of cochlear hair cells from aminoglycoside toxicity ex vivo. To exclude the possibility of ADSCs' penetration into cochlea explants, the cochlear explants were separated from ADSCs by culture mesh inserts to avoid direct contact. We demonstrated that co-culture with ADSCs significantly promotes the survival of cochlear hair cells in explants against neomycin toxicity. We then measured the protein levels of several growth and trophic factors in the culture media of ADSCs using ELISA, revealing the presence of IGF-1, VEGF, TGF-β1, NGF, and robust amounts of HGF, which has protective effects on cochlear hair cells against neomycin toxicity[9] and noise-induced damage.[10] IGF-1 also demonstrates important protective effects on cochlear hair cells against noise trauma[6,7] and ischemic injury.[8] VEGF is associated with attenuation of noise-induced damage on cochlear hair cells,[17] whereas TGF-β1 exhibited hair cell protection against aminoglycosides in combination with glial cell line-derived neurotrophic factor.[5] In short, most ADSC-secreted growth factors presented in the current study have the potential to protect cochlear hair cells, which strongly supports our hypothesis about the paracrine effects of ADSCs.

In comparison with previous studies showing the direct effects of growth factors on hair cell protection, the levels of growth factors demonstrated in the present study were considerably low. HGF levels (6 ng/mL) were the highest of all growth factors measured in this study, but were still less than HGF levels reported to demonstrate protective effects on cochlear hair cells in explant cultures against neomycin toxicity.[9] Although we used the same explant culture system in both studies, our previous work showed significant protective effects of as much as 20 ng/mL HGF on cochlear hair cells against 1 mM neomycin, with no protection provided by 4 ng/mL

HGF.[9] Based on these findings, we speculate that the protection observed in the present study was achieved by the synergistic effects of multiple growth factors secreted from ADSCs, although we cannot exclude the possibility that the recombinant HGF we used in the previous report had weaker activity than HGF ADSCs secreted in this study because of the difference in the post-transcriptional regulation. Indeed, TGF-β1 enhanced the protective effects of glial cell line-derived neurotrophic factor on cochleae against aminoglycosides,[5] which supports our synergistic hypothesis.

We also measured the levels of several cytokines in ADSC culture media, because cytokines derived from ADSCs could exert protective effects on hair cells. In the present study, ELISA analysis demonstrated the secretion of MCP-1 from ADSCs to the culture media. MCP-1 is a small cytokine belonging to adipocytokines that is also known as chemokine (C-C motif) ligand 2. MCP-1 displays chemotactic activity to monocytes and macrophages. Recently, MCP-1 was also known to play an important role in the regulation of metabolism[18] and in the pathogenesis of neurodegenerative diseases.[19] In the inner ear, the involvement of MCP-1 has been demonstrated in the process of inflammatory responses following exposure to otitis media pathogens[20] and acoustic overstimulation.[21] In addition, previous studies have suggested that migration of macrophages into cochlear tissue following traumatic insults contributes to the protection of cochlear tissues[21,22] However, direct effects of MCP-1 on hair cells have not been elucidated. Therefore, it is unclear whether MCP-1 secreted from ADSCs plays positive or negative roles in hair cell protection against neomycin toxicity. However, the present finding indicates that ADSCs could modulate inflammatory responses in the cochlea under pathological conditions via paracrine of MCP-1.

The cell transplantation approach can be advantageous at some points when we compare it with local application of growth factors. First, transplanted cells can keep secreting growth factors for a long period. Second, the secretion volume can be regulated by cross-talk with surrounding cells including damaged cells. Third, the cells may secrete unknown growth factors that have protective effect on hair cells. Fourth, the growth factors transplanted cells secrete can have higher activity than recombinant growth factors because they were regulated by physiological post-transcriptional modifications. Although these are advantages of the cell transplantation approach, it requires surgical procedure for opening the cochlear fluid space. One possible application of ADSC transplantation is hybrid cochlear implantation,[23] where low frequency auditory stimuli are transmitted to the auditory primary neurons by the remaining hair cells, and high frequency auditory stimuli are transmitted to the auditory primary neurons by direct electrical stimulation via cochlear implant devices. In the case of hybrid cochlear implantation, the preservation of residual hearing at low frequency regions is critical. It is therefore important to protect cochlear hair cells from invasive surgery. During cochlear implant surgery, the cochlear bony wall is opened to insert a cochlear implant electrode; at this stage, therapeutic cells could be transplanted into the cochlea. Because the capacity of ADSCs to protect cochlea hair cells was demonstrated in this study, ADSCs are expected to be applied to clinical use in case of hybrid cochlear implantation in future.

CONCLUSION

The present findings demonstrate that ADSCs have the capacity to protect cochlear hair cells via paracrine effects, suggesting that ADSC transplantation into cochleae may be a useful strategy for the protection of cochlear hair cells in vivo. As a next step, we will examine the effects of ADSC transplantation for cochlear protection using animal models in vivo.

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Insulin-like growth factor 1 protects vestibular hair cells from aminoglycosides

Nakarin Angunsri[a,b], Akiko Taura[a], Takayuki Nakagawa[a], Yushi Hayashi[a], Shin-ichiro Kitajiri[a], Eigo Omi[b], Kazuo Ishikawa[b] and Juichi Ito[a]

This study investigated the therapeutic potential of insulin-like growth factor-1 (IGF-1) for vestibular hair cells using explant cultures of mouse utricles. After incubation with the ototoxic drug gentamicin, explants from neonatal mouse utricles were cultured in medium containing IGF-1 at various concentrations. Histological evaluation revealed significant increases in the number of surviving hair cells cultured with IGF-1 at concentrations reflecting a clinical setting. Immunostaining for trio-binding protein and espin showed the maintenance of functional structures in hair bundles at the apex of surviving hair cells. An FM1-43 assay indicated the presence of mechanoelectrical transduction channels in surviving hair cells. These findings indicate that IGF-1 may protect the functionality of vestibular hair cells against drug-induced injury. *NeuroReport* **22:38–43 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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[a]Department of Otolaryngology and Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto and [b]Department of Otolaryngology and Head and Neck Surgery, Akita University School of Medicine, Akita, Japan

Correspondence to Takayuki Nakagawa, MD, PhD, Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyoku, Kyoto 606-8507, Japan
Tel: +81 75 751 3346; fax: +81 75 751 7225;
e-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

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Introduction

The peripheral vestibular apparatus consists of two otolith organs and three semicircular canals. Vestibular sensory epithelia report the magnitude and direction of angular and linear motion of the head to the central nervous system. Normal sensory processing through the vestibular system is largely unconscious, and we become aware of it only when it malfunctions. Dysfunction of the peripheral vestibular apparatus has an incapacitating influence on every aspect of our lives, and drug-induced damage can cause serious disability as it often leads to bilateral dysfunction.

Hair cells are the primary mechanotransducers of the peripheral vestibular apparatus. A bundle of enlarged microvilli, stereocilia, is present at the apex of each hair cell, and the tilt of these bundles induces the opening of mechanoelectrical transduction (MET) channels, which is the primary step in the sensing of head movement by the vestibular system. The survival of hair cells is therefore crucial for the maintenance of vestibular functions. In mammalian inner ears, cochleae have limited capacity for spontaneous regeneration. Although mammalian vestibular epithelia can undergo hair cell regeneration [1–5], this is not sufficient for functional recovery [1,4–6].

The protection of hair cells is therefore a practical strategy for the treatment of the mammalian vestibular epithelium. Earlier studies have indicated the potential of growth factors, including insulin-like growth factor-1 (IGF-1), for the protection and regeneration of vestibular hair cells [7–11]. This study focused on IGF-1 and examined these effects using explant culture systems with special attention to the functionality of hair cells.

Methods

Animals

ICR mice (Japan SLC Inc., Hamamatsu, Japan) were maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Japan. Experimental protocols were approved by the Animal Research Committee of Kyoto University Graduate School of Medicine (MedKyo10119), and complied with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Explant culture

Postnatal day 3 mice were deeply anesthetized with sevoflurane and decapitated. The temporal bones were dissected out and the utricles were removed from the surrounding tissue in 0.01 M phosphate-buffered saline, pH 7.4, which was supplemented with 0.2% glucose. The otoconial membranes were gently removed with a fine needle. Explants of utricle sensory epithelia were placed intact on type I collagen-coated cover glass (Iwaki, Tokyo, Japan) and maintained in 24-well culture plates (Iwaki) in Dulbecco's modified Eagle's medium (Invitrogen, Eugene, Oregon, USA), supplemented with 6 g/l glucose

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