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Short report

Age-dependent modifications of expression level of VEGF and its receptors in the inner ear

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Abstract

The mechanisms responsible for age-associated hearing loss are still incompletely characterized. In this study, we used a murine model of age-dependent hearing loss and evaluated whether this condition is associated with vascular modifications of the structures of the inner ear. We used old C57BL/6J mice that are affected by rapid and severe age-related hearing loss, and analyzed the expression pattern of vascular endothelial growth factor (VEGF), a prototypical angiogenic cytokine, and its receptors Flt-1 and Flk-1 in the inner ear. We report for the first time morphological and quantitative data about the expression of these crucial angiogenic molecules in the murine cochlea. We also show that in this animal model, cochlear VEGF expression is significantly reduced as a function of age. Our findings provide new evidence of possible interdependent relationships between aging, VEGF, and presbycusis, suggesting that vascular abnormalities might play a role in aging-associated hearing loss, with potentially important fundamental and clinical implications.

Keywords: Hearing loss; Cochlea; Aging; VEGF; Flt-1; Flk-1

1. Introduction

Presbycusis is the most common cause of hearing loss in aged subjects. Old C57BL/6J mice are characterized by rapid and early auditory decline and represent an interesting animal model of presbycusis (McFadden et al., 1997; Johnson et al., 1997). At 6 months of age, these animals show a significant hearing loss for high-frequencies (<20 kHz), that eventually involves lower frequencies at 12 months of age. In 15-months-old mice, a profound pan-tonal hearing loss (<80 dB SPL) (Li and Borg, 1991) can be detected. These functional deficits are strongly associated with degenerative histopathological changes. Between 6 and 12 months of age, the basal turn of the cochlea and in particular, the outer pillar cells of the organ of Corti, degenerates. The loss of outer hair cells (OHCs) is early and severe, while inner hair cells (IHCs) exhibit a milder and later loss. At this time-point, the degenerative process also involves the neuroepithelium and the supporting cells. At 2 years of age, no recognizable structures are present in the basal turn of the organ of Corti of C57BL/6J mice. Regarding spiral ganglion cells, a pronounced loss occurs with age, with an almost complete loss in the basal turn during the second year of life (Henry and Chole, 1980; Hunter and Willott, 1987).

Among the several morphological changes described in the inner ear of aged C57BL/6J mice, those involving the vascular structures and in particular, the stria vascularis (SV), have been less extensively investigated. Our group has recently demonstrated that aging is associated with vascular network changes in C57BL/6J mice, the main modifications consisting of reduced capillary density and vascular diameter (Di Girolamo et al., 2001). These data suggest that in C57BL/6J mice, vascular modifications of the inner ear may be involved in age-related hearing loss.

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This hypothesis is consistent with the notion that aging is characterized by profound alterations of the metabolic phenomena regulating morphology and function of vascular structures. In particular, it has been shown that angiogenesis, defined as the growth of new blood vessels from a pre-existing vascular network, is deficient in old organisms, as a result of inherent inability to upregulate specific angiogenic growth factors in response to angiogenic stimuli. Indeed, reduced expression of vascular endothelial growth factor (VEGF), a prototypical angiogenic agent, has been reported in the ischemic hindlimb of aged mice and rabbits (Rivard et al., 1999; Sadoun and Reed, 2003). VEGF is crucial for endothelial function and metabolism and plays a crucial role in the promotion of angiogenesis and the maintenance of an efficient tissue vascularization. Therefore, its reduced expression might result in profound functional alterations in a number of organs and tissues, including those forming the inner ear.

The aim of this study was to evaluate whether differences in the expression of VEGF and its functional receptors exist in the inner ear of young and old mice. We found that the expression levels of VEGF and its functional receptors were modified in the inner ear during aging of C57BL/6J mice.

2. Methods

Three-months- (group A, $N = 6$) and 24-months-old (group B, $N = 6$) C57BL/6J mice of either gender, weighing approximately 20 g, were used. All animals were deeply anaesthetized via an intramuscular injection of 200 μl of a saline solution containing 0.05 mg/ml of metedomidine and ketamine. All the experiments were conducted in accordance with the Institutional Animal Care and Use Committee of our University Hospital.

For immunohistochemical studies, in three animals of group A and three animals of group B, the chest was opened, an incision was made in the right atrium, and 1 ml of 4% paraformaldehyde was delivered systemically by intracardiac injection. The temporal bones of each animal were removed, the bulla was opened, the cochleae were immersed in the same fixative for 3 h, and then kept at 4 °C overnight. Cochleae were decalcified by using a 10% EDTA solution over a period of 3 days. Tissues were embedded in paraffin and cut into cross-sections. The following primary antibodies were used: rabbit polyclonal anti-mouse VEGF (Santa Cruz Biotechnology), rabbit polyclonal anti-mouse Flt-1 (Santa Cruz Biotechnology), and rabbit polyclonal anti-mouse Flk-1 (Santa Cruz Biotechnology). A biotinylated goat anti-rabbit immunoglobulin was used as secondary antibody (Signet Labs). Negative control slides were prepared by substituting preimmune rat serum. Intensity and immunostaining was evaluated by two blinded independent operators and classified as follows: + (faint), ++ (moderate), and +++ (intense), as previously described (Palozza et al., 2002).

For Western blotting analysis, three animals of the group A and three animals of the group B were decapitated without perfusion. The bulla was opened and the cochleae were immediately frozen in liquid nitrogen and stored at − 80 °C until used. Samples were then homogenized in lysis buffer (100 mM potassium phosphate, 0.2% Triton X-100) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Total protein extracts were quantified by the BCA protein assay kit (Pierce, Rockford, IL). In each experiment, equal amounts of reduced protein extracts were separated on 15% polyacrylamide gels (Bio-Rad, Rockville Center, NY) and electroblotted on a nitrocellulose membrane, which was blocked with 5% nonfat dry milk in 0.2% Tween PBS (T-PBS). Samples were then probed with VEGF goat polyclonal antibody (Sigma, 1:1000 dilution) and with polyclonal Flt-1 and Flk-1 antibody for 2 h at room temperature. The membrane was washed three times in T-PBS and then incubated with anti-goat (1:10,000) horseradish peroxidase IgG for 1 h. Antigen–antibody complexes were visualized after incubation for 1 min with enhanced luminescence reagent (Amersham Pharmacia Biotech) at room temperature, followed by exposure to hyperfilms (Amersham Pharmacia Biotech). Equal protein loading among individual lanes was confirmed after stripping the membranes with ImmunoPure elution buffer (Pierce) by reprobing the membranes with an α-tubulin mouse monoclonal antibody at 1:1000 dilution (Calbiochem).

3. Results

3.1. Immunoblotting

Expression of VEGF was first analyzed by Western blotting in the whole cochlea. We found that this protein is strongly expressed in young mice, whereas its expression is substantially reduced in old animals. The same tissue extracts were used to evaluate the expression of the VEGF receptors, Flt-1 and Flk-1. Flt-1 was more strongly expressed than Flk-1 in the cochleae of both young and old mice. However, no differences in the expression of either receptors were found as function of age (Fig. 1).

3.2. Immunohistochemical study

In order to identify which cochlear structures express VEGF and its receptors in young and old mice, a series of immunohistochemical studies was performed.

In young mice, VEGF was detected in the SV, the spiral ligament, and the spiral ganglion cells (Fig. 2). In contrast, old mice exhibited a substantially weaker VEGF immunoreactivity in either the SV, the spiral ligament, or the spiral ganglion cells (Fig. 2).

The organ of Corti of young mice, including the supporting cells, the IHCs, and the OHCs, was strongly

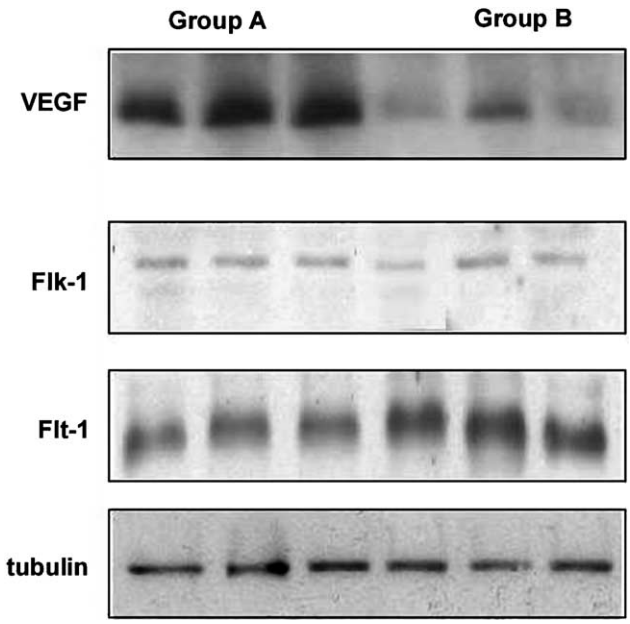

Fig. 1. Western blotting for VEGF and its receptors in the cochlea of young and old mice. The expression level of VEGF protein is significantly reduced in old mice (group B), compared to young animals (group A). Flt-1 has a higher expression than Flk-1. Both VEGF receptors have similar expression levels in group A and B mice.

immunopositive for VEGF (Fig. 3 and data not shown). Profound reduction of VEGF immunostaining was instead found in the organ of Corti of old mice (Fig. 3). In these animals, it was also possible to observe a clear degeneration of the supporting cells, along with loss of IHCs and OHCs and severe modifications of the neuroepithelium structures (data not shown). A quantification of VEGF expression in the different cochlear regions of young and old mice, as inferred by immunohistochemical analyses, is shown in Table 1.

Flt-1 and Flk-1 immunostaining was used to identify the cochlear structures that express these VEGF receptors. This analysis showed that in young mice, Flt-1 is expressed in the cochlear regions that are also immunopositive for VEGF (Fig. 4a–c). In contrast, in mice included in the group B, Flt-1 immunopositivity was weak to be absent in the organ of Corti (data not shown), while it was substantially unchanged in the SV and the spiral ligament (Fig. 4d). Regarding Flk-1 expression, this VEGF receptor was absent in the IHCs and OHCs of the organ of Corti (data not shown), while it was detected in the spiral ganglion cells, the SV, the modiolus, and the basilar membrane of young mice (Fig. 4e–g and data not shown). In the group B mice, Flk-1 immunostaining was substantially similar to that observed in group A, with the exception of a slight enhancement at the level of the SV (Fig. 4h). A quantification of Flt-1 and Flk-1 immunoreactivity in all the cochlear structures is shown in Table 2.

4. Discussion

VEGF is an endothelial cell (EC)-specific mitogen, with crucial functions in vascular metabolism. VEGF has indeed

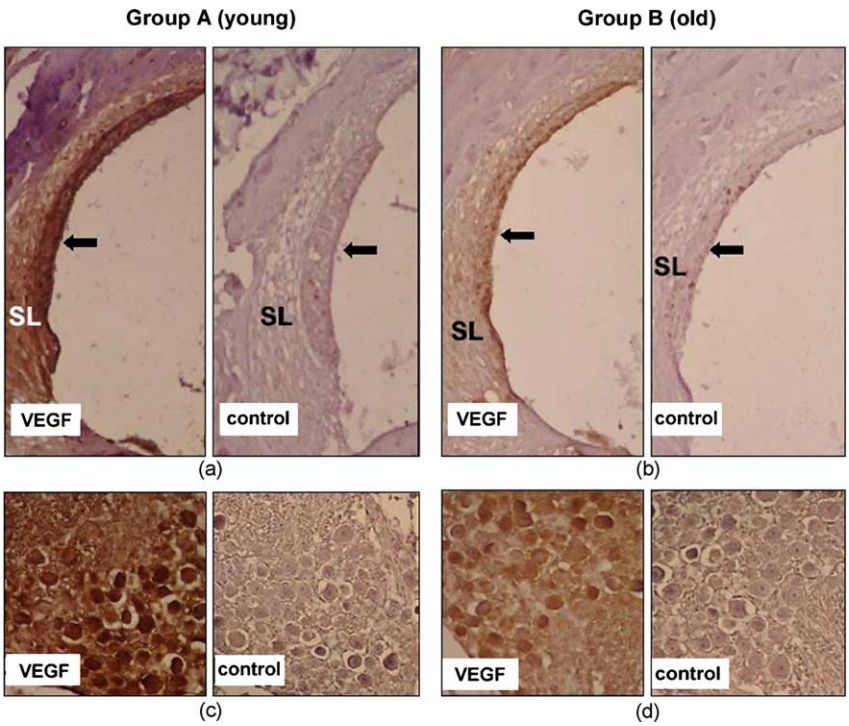

Fig. 2. VEGF immunoreactivity in the stria vascularis, the spiral ligament, and the spiral ganglion cells of young and old mice. VEGF is strongly expressed in the stria vascularis (arrow) and the spiral ligament (SL) of young mice (a). Old mice exhibit lower VEGF immunoreactivity in either the stria vascularis (arrow) and the SL (b). VEGF is more strongly expressed in the spiral ganglion cells of young mice (c) than in those of old animals (d).

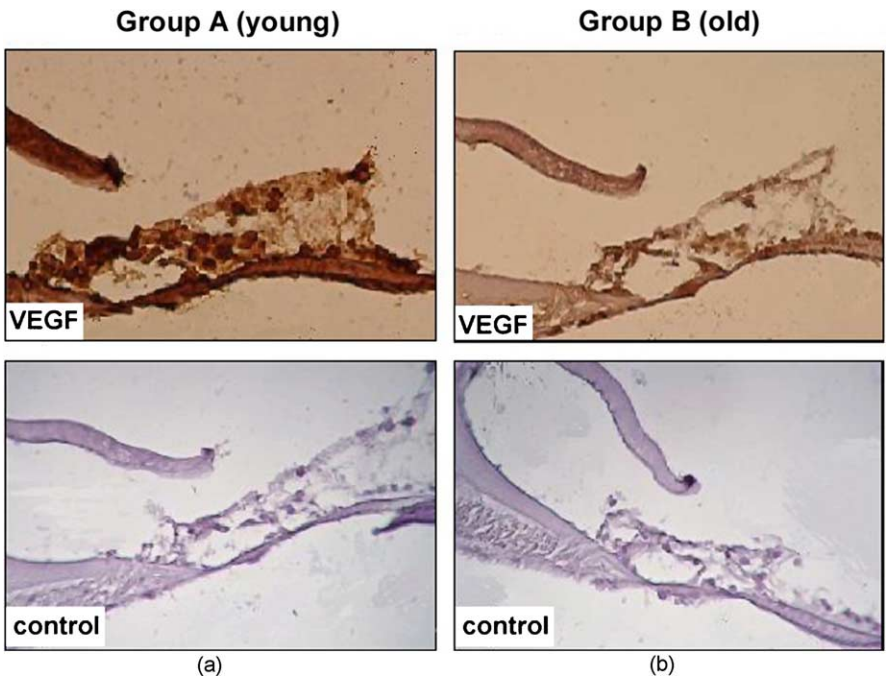

Fig. 3. VEGF immunoreactivity in the organ of Corti of young and old mice. Immunoreactivity for VEGF is stronger in the organ of Corti of young mice (a) than in that of old mice (b).

important effects on vascular permeability, EC migration, vessel formation and relaxation, and apoptosis. These multiple properties are responsible for its potent angiogenic ability. VEGF acts by binding to two tyrosine-kinase receptors, VEGF receptor-1 (VEGFR-1 or Flt-1) and VEGF receptor-2 (VEGFR-2 or KDR or Flk-1), which are almost exclusively expressed in ECs (Ferrara et al., 1992). The expression of VEGF is increased in response to hypoxia, but is also augmented by activated oncogenes and a variety of other cytokines.

There is now increasing appreciation of the fact that reduced vascularization and endothelial dysfunction may be important pathogenetic mechanisms in many otologic disorders, including noise-induced hearing loss (Attanasio et al., 2001), endolymphatic hydrops, and presbycusis (Thomopoulos et al., 1997; Prazma et al., 1990). VEGF itself has been implicated in the pathophysiology of several disorders of the central and peripheral nervous system and its possible role in processes leading to cochlear dysfunction and hearing loss may be hypothesized (Carmeliet and Storkebaum, 2002). Nevertheless, very little has been done to understand the function and expression of VEGF signaling pathway in the inner ear. Indeed, the expression pattern of VEGF and its receptors in the inner ear has been only described in guinea pigs (Hess et al., 2000; Michel et al., 2001) and during otitis media in the rat (Chae et al., 2003).

Our study reports for the first time quantitative and anatomical data regarding the expression of VEGF and its functional receptors in the murine inner ear. We also provide evidence that aging is associated with significant modifications in the cochlear expression pattern of VEGF. Indeed, we observed the presence of VEGF in the spiral ganglion cells, the cells of spiral ligament, the supporting cells, the inner and OHCs, and the SV. In aged animals, VEGF expression is instead reduced in all the cochlear regions and in particular, in the SV. Another novel finding reported in this study is the expression pattern of the VEGF receptors Flt-1 and Flk-1. The first is expressed in the same region of VEGF, while Flk-1 is detected in the SV, the spiral ganglion cells, the spiral ligament, the modiolus, the basilar membrane, and the supporting cells of the organ of Corti. In aged animals, both receptors are more expressed in the SV.

Why VEGF expression is reduced in the cochlear structures of aged mice remains to be elucidated. However, several mechanisms have been suggested as potentially responsible for the impairment of angiogenesis-associated

Table 1
Quantification of VEGF expression by immunostaining in different cochlear regions, in young and old mice

Cochlear region	VEGF expression	
	Group A	Group B
Modiolus	+++	+
Spiral ganglion	+++	+
Spiral ligament	+++	+
Basilar membrane	+++	+
Supporting cells	+++	+
IHCs	+++	+
OHCs	+++	+
Stria vascularis	+++	++

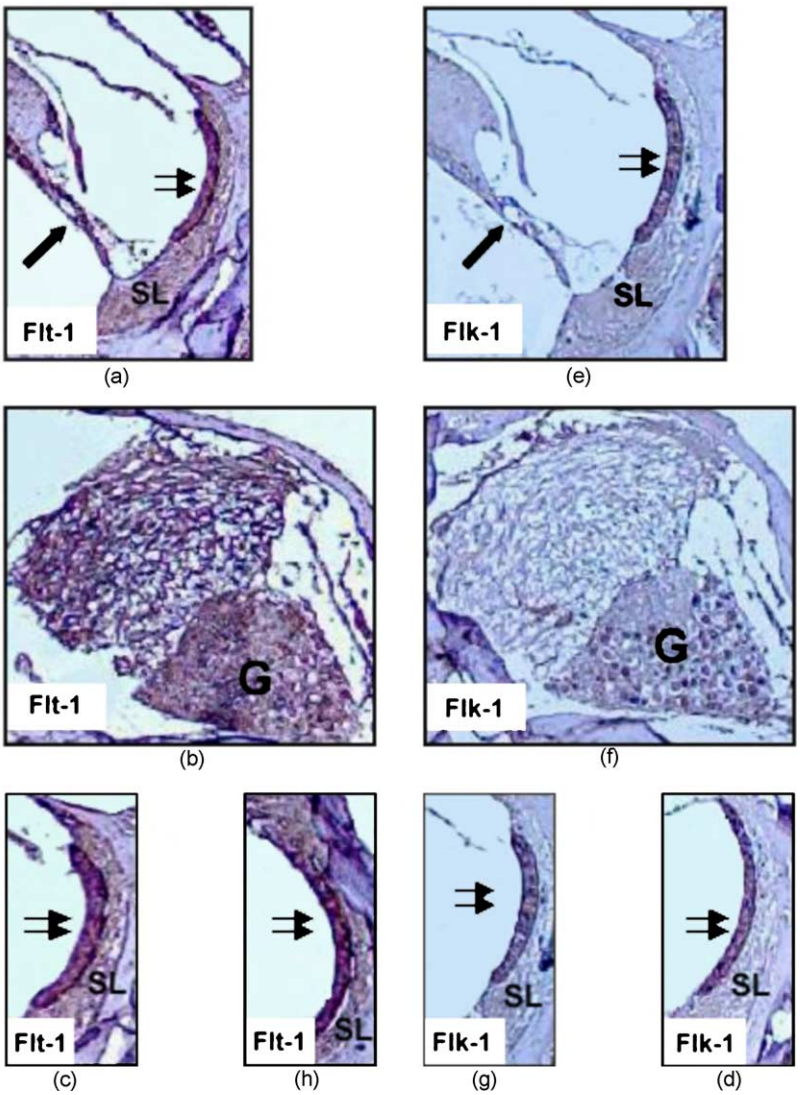

Fig. 4. Flt-1 and Flk-1 immunoreactivity in the cochlea of young and old mice. Flt-1 is expressed in the organ of Corti (arrow), the spiral ganglion cells (G), the stria vascularis (double arrow), and the spiral ligament (SL) of young mice (a–c). A similar Flt-1 immunoreactivity can be detected in the stria vascularis (double arrow) of old mice (d). Flk-1 is not expressed in the organ of Corti (arrow), is very weakly expressed in the spiral ligament (SL), and is present in the spiral ganglion cells (G) and the stria vascularis (double arrow) of young mice (e–g). The stria vascularis (double arrow) of old mice is also immunopositive for Flk-1 (h).

processes in aging. First, it has been shown that aged animals display reduced activity of the hypoxia-inducible factor-1, resulting in impaired ability to upregulate VEGF in response to ischemic stimuli (Rivard et al., 2000). Second, aging has been associated with multiple alterations in matrix composition and inflammatory response that are crucial phenomena in physiologic and pathologic angiogenesis (Sadoun and Reed, 2003). Third, senescence also influences plasticity and migratory capacities of stem cells and progenitor cells, thus reducing their potential contribution to neoangiogenic processes (Dimmeler and Vasa-Nicotera, 2003). Finally, VEGF-induced reendothelialization is impaired in aged mice, suggesting that also ischemia-independent mechanisms of VEGF upregulation are reduced as function of age (Gennaro et al., 2003).

Table 2
Quantification of Flt-1 and Flk-1 expression by immunostaining in different cochlear regions, in young and old mice

Cochlear region	Flt-1 expression		Flk-1 expression	
	Group A	Group B	Group A	Group B
Modiolus	+	++	+	+
Spiral ganglion	+++	+++	++	++
Spiral ligament	+++	+++	+	+
Basilar membrane	++	+++	++	++
Supporting cells	++	+	++	++
IHCs	++	+	−	−
OHCs	++	+	−	−
Stria vascularis	+	+++	++	+++

While VEGF expression is significantly reduced in the inner ear of old mice, the expression of VEGF receptors is not substantially different between old and young animals. The functional significance of this finding remains to be elucidated. However, it is important to note that while it has been clearly demonstrated that aging reduces VEGF expression and impairs VEGF upregulation, no data are available in the literature about the effects of aging on the expression levels of Flt-1 and Flk-1. Under this respect, this study is the first providing evidence that these VEGF receptors are normally expressed in aging mice. Although, this finding is limited to the inner ear and need confirmation in other organs, it suggests that during aging, the VEGF downstream signaling pathway is still integral and potentially functional. This hypothesis is indeed consistent with previous results indicating that aging does not preclude augmentation of collateral vessel development upon stimulation with appropriate agonists and inducers (Rivard et al., 1999; Gennaro et al., 2003), with potentially important therapeutic implications.

In conclusion, this study provides evidence that age-related hearing loss and histopathological changes of the organ of Corti occur in association with important alterations of the vascular pattern and reduced expression of VEGF in the cochlea. These findings suggest that vascular dysfunction might play a role in aging-associated hearing loss.

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