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Preclinical report

Expression of inducible nitric oxide synthase (iNOS/NOS II) in the vestibule of guinea pigs after the application of cisplatin

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It is well known that the anti-cancer drug cisplatin has an ototoxic property; however, the details are not yet evident. In this study, the expression of inducible nitric oxide synthase (iNOS/NOS II) in the vestibule of guinea pigs after i.p. injections of cisplatin was examined immunohistochemically. Three days after the injection of cisplatin (10 mg/kg) or placebo, animals were sacrificed. Then the temporal bones were removed and subjected to immunohistochemical studies for iNOS. In the cisplatin group, iNOS was detectable, whereas the tissue in the control group was negative for iNOS. The vestibule, the wall of blood vessels and the vestibular ganglion cells showed immunoreactivity for iNOS. It is known that iNOS catalyzes an inadequate quantity of NO under pathological conditions. Increased NO levels lead to inner ear dysfunction. Therefore, our results indicate that iNOS could also mediate the vestibulo-toxicity of cisplatin.

Key words: Cisplatin, inducible nitric oxide synthase, ototoxicity, vestibule.

Introduction

Nitric oxide (NO) is reported to possess various bioactivities. NO is catalyzed from L-arginine by the action of NO synthase (NOS). NOS has three isoforms, two of them are constitutive Ca^{2+} dependent: (i) endothelial NOS (eNOS/NOS III) and (ii) brain NOS (bNOS/NOS I). Both constitutive NOS (cNOS) release small amounts of NO to regulate physiological conditions. In the cardiovascular system NO mediates blood flow and pressure through action on the smooth muscle cells of blood vessels. bNOS is detected mainly in the central nervous system and bNOS-generated NO works as a neurotransmitter.[1–3] By contrast, Ca^{2+}-independent iNOS catalyzes (once expressed) uncontrolled 100- to 1000-fold higher amounts of NO. NO reacts with superoxides and produces peroxynitrites ($OONO^-$) which have strong oxidative effects.[4,5] This is evidence for cytotoxic effects on tissues. In the inner ear, iNOS is detected under pathological conditions, particularly inflammation, noise exposure or endolymphatic hydrops (unpublished data).[6–8] These reports suggest that inadequate quantities of NO catalyzed by iNOS and free radical species are associated with various pathological conditions of the inner ear.

The oto- and vestibulo-toxicity of the anti-cancer drug cisplatin is a well-known side effect.[9] However, details of such toxicity are not yet evident. Recent reports indicate that cisplatin increases the activity of iNOS in the kidney and liver,[10] and free radical species are detected in the inner ear after the application of cisplatin.[11] From these reports, it is presumed that iNOS-mediated NO plays a part in the vestibulo-toxicity of cisplatin.

In this study we examined the immunoreactivity for iNOS in the vestibule and discussed the mechanism of the vestibulo-toxicity after cisplatin application.

Materials and methods

Materials

Twelve guinea pigs (250–350 g) were used in this study. All animals were confirmed to have a positive Preyer's reflex and were microscopically examined to be free from otitis media. Animals were anesthetized

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with 5% ketamin hydrochloride (50 mg/kg body weight) and 2% xylazin hydrochloride (10 mg/kg body weight) before all procedures. The animals were divided into a cisplatin and a control group. In the CDDP group (n=6) 10 mg/kg body weight of cisplatin (0.5 mg/ml; ASTA Medica, Frankfurt, Germany) dissolved in physiological saline (NaCl 0.9%) was injected i.p. In the control group (n=6), physiological saline (10 ml/kg, NaCl 0.9%) was injected. This investigation was approved by the Ethical Committee of Animal Experimentation (Bezirksregierung Köln/Germany, permit 23.203.2 K42 3/98).

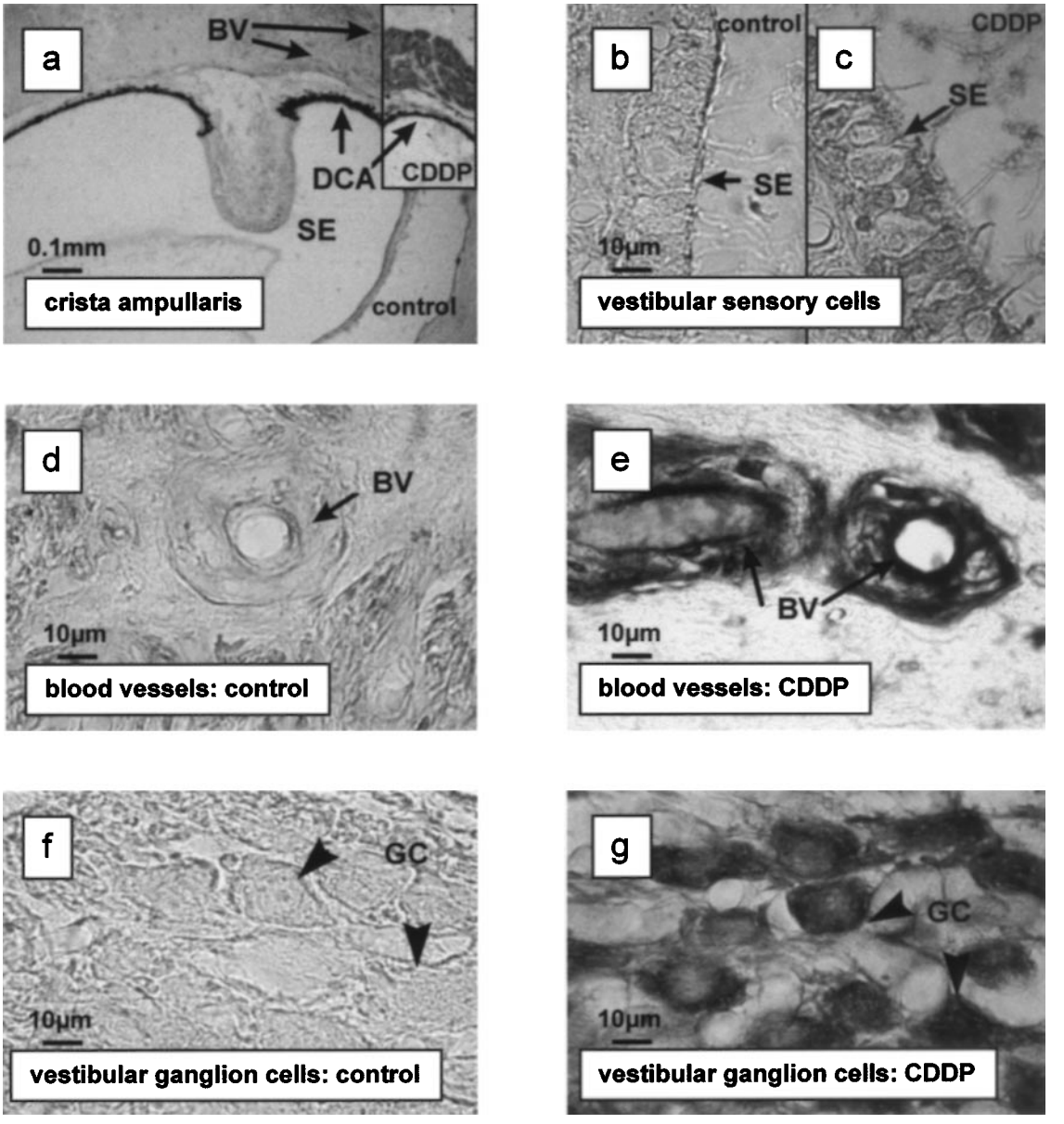

Figure 1. Paraffin sections of the vestibule, 8 μm. Immunohistochemistry, anti-iNOS. (a) The crista ampullaris is shown. The natural pigmentation was observed in the dark cell area (DCA); however, no immunoreaction is evident in the control group. On the right top, a part of the cisplatin-treated vestibule is shown. The blood vessels (BV) in the vestibular bone displayed iNOS expression. There were no significant changes in the DCA between the control and cisplatin group, $\times$25. (b) Control group, the vestibular sensory epithelium (SE) is shown. There were no apparent immunoreactivities for iNOS, $\times$250. (c) Cisplatin group, vestibular hair cells and supporting cells also showed no apparent immunoreactivity for iNOS, $\times$250. (d) Control group, the wall of BV showed no immunoreactivities for iNOS, $\times$250. (e) Cisplatin group, the wall of BV showed a strong reactivity, $\times$250. (f) Control group, the vestibular ganglion cells (GC, arrow head) are shown. No immunoreactivities were detected. $\times$250. (g) The perikarya of the vestibular GC (arrow head) displayed iNOS immunolabeling, $\times$250.

Immunohistochemical examination

All animals were sacrificed 3 days after the injection of each solution. The tissues were fixed via cardiac perfusion with 4% paraformaldehyde (pH 7.4) after flushing out the blood with 0.1 M PBS. The cochleas were incubated in the same fixative overnight. Decalcification was performed with 10% EDTA solution in Tris at pH 7.4 for 5 days. Subsequently, the tissues were embedded in paraffin. Each paraffin-embedded specimen was sectioned at a thickness of 8 μm with a microtome (HM360: Microm, Walldorf, Germany). The paraffin was removed by immersion in a graded series of ethanol. Then the sections were immersed in 3% H_2O_2 for 20 min, followed by 0.25% Triton X for 10 min. Subsequently they were incubated with the first antibody to iNOS at 1:1000 dilution (rabbit polyclonal antibody, SA200; Biomol, Hamburg, Germany) overnight. After rinsing with 0.1% Tris–PBS solution (pH 7.4) and treatment with 3% normal goat serum, the sections were incubated in the second antibody at 1:400 dilution (anti-rabbit; Dako, Glostrup, Denmark) for accentuation. The reaction was developed with a horseradish peroxidase complex at 1:100 dilution for 1 h (Amersham, Little Chalfont, UK) and nickel-enhanced DAB (Sigma, St Louis, MO). Additionally, tissues of the bacterial endotoxin lipopolysaccharide induced shock and the squamous cell carcinoma were taken for the positive control to iNOS.

Results

Immunohistochemical expression of iNOS

In the control animal group, the structures of the vestibules which were positive for iNOS expression were not found in the crista ampullaris (Figure 1a), the sensory epithelium (Figure 1b), vestibular ganglion cells (Figure 1f) and the wall of the blood vessels in the vestibular bone (Figure 1d).

Immunoreactivities for iNOS were detected 3 days after the application of cisplatin in all animals. The wall of blood vessels in the vestibular bone showed a strong iNOS reactivity (Figure 1a and e). Some perikarya of the vestibular ganglion cells also showed iNOS expression (Figure 1g). In contrast, vestibular hair cells, supporting cells and nerve showed no significant iNOS staining (Figure 1c). The structure of sensory epithelium seemed to be maintained under light microscopy. In the dark cell area there were no significant changes between the control and cisplatin group (Figure 1a).

Discussion

The oto-toxicity of cisplatin is a well-known effect.[9,12–15] The sensory organs of the inner ear are composed of both the cochlea and the vestibule. However, there are few reports concerning vestibular disturbance.[16,17]

Our data show that after the application of cisplatin, iNOS was expressed in the vestibule. iNOS immunoreactivity was particularly detected in the wall of blood vessels and vestibular ganglion cells, not in the sensory epithelium and supporting cells. In the cochlea, cisplatin is known to cause damage to the stria vascularis, which is the main energy source for the cochlea, the sensory cells and supporting cells.[9,18] These changes are thought to induce cochlear dysfunction. On one hand, Srivastava *et al.*[10] reported that cisplatin significantly increases the activity of iNOS and enhances lipid peroxidation in the kidney and liver. iNOS catalyzes inadequate amounts of NO. NO reacts with superoxides and produces $OONO^-$.[4,5] $OONO^-$ rapidly dissociates to the hydroxyl radical. Damaged cells are stimulated to release cytokines. Ranjan *et al.*[19] reported that cisplatin and cytokine-treated macrophages produced higher amounts of NO than cisplatin or cytokines alone. NO could also activate the negative feedback mechanism on *N*-methyl-D-aspartate (NMDA) receptors (one of excitatory receptors) and lead to neuronal block in the vestibule.[20] These reports show that inadequate high amounts of NO and free radical species have cytotoxic properties.

Similar to the cochlea, it is likely that the cytotoxic effect on the vestibule is partly due to iNOS catalyzed NO and superoxide species. However, the sensory epithelium and the supporting cells in the vestibule exhibited no apparent immunoreactivity for iNOS or morphological changes under light microscopy in our study. Administrated cisplatin exerts its effects on the vestibule via blood vessels and/or endolymph. Suzuki *et al.*[21] showed that the basement membrane anionic sites in the cochlea were more affected by cisplatin than the vestibules. Our result showed that no apparent iNOS expression was detected in the dark cell area, which is thought to have a similar role to the stria vascularis.[22] From these reports and our results, it is reasonable that there might be a different vulnerability against cisplatin between the vestibule and the cochlea, as clinical experience has shown.

In conclusion, our results suggest that the expression of iNOS and consecutive unphysiological high NO levels contribute to the vestibulo-toxicity of cisplatin.

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