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Preclinical report

Nitric oxide synthase inhibitor reduces the apoptotic change in the cisplatin-treated cochlea of guinea pigs

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Cisplatin is known to cause inner ear damage. The role of nitric oxide (NO) in the cochlea of the guinea pigs after injections of cisplatin or a combination of cisplatin and NO synthase (NOS) inhibitor [N^G-nitro-L-arginine methyl ester (L-NAME)] i.p. was examined by means of immunohistochemistry. Three days after injection, the cochleas were examined immunohistochemically for single-stranded DNA (ssDNA). We found that ssDNA was expressed in the stria vascularis and spiral ganglion cells of the cisplatin-treated cochlea. In the L-NAME/cisplatin-treated cochlea, the number of cells that exhibited positive staining for ssDNA was markedly reduced. High NO levels lead to inner ear dysfunction under pathological conditions. Our results indicate that NO mediates the ototoxicity of cisplatin. [© 2000 Lippincott Williams & Wilkins.]

Key words: Anti-NOS drug, apoptosis, cisplatin, nitric oxide, ototoxicity, single-stranded DNA.

Introduction

The anticancer drug cisplatin shows severe side effects, e.g. renal toxicity, myelosuppression and ototoxicity. Clinical studies revealed inner ear disturbance following the admission of cisplatin, such as hearing loss, decreased speech discrimination, etc. Recent reports indicate that cisplatin increases the activity of inducible nitric oxide synthase (iNOS/NOS II)[1] and iNOS catalyzed large amounts of nitric oxide (NO) that takes part in the cytotoxicity of cisplatin.[2]

NO is known to have various biological activities. NO is catalyzed by the action of NOS. Among NOS, three isoforms were detected. Constitutive NOS (cNOS), endothelial NOS (eNOS/NOS III) and brain NOS (bNOS/NOS I) release small amounts of NO to maintain homeostasis under physiological conditions. In the cardiovascular system, NO acts as an endothelium-derived relaxing factor, regulating blood flow through its action on the smooth muscle cells of blood vessels. bNOS is detected mainly in the central nervous system as a neurotransmitter.[3] By contrast, iNOS catalyzes 100- to 1000-fold higher amounts of NO. In the cochlea, iNOS activities are also detected under pathological conditions, e.g. inflammation, noise exposure, endolymphatic hydrops or administration of ototoxic drugs.[4–7] These reports suggest that inadequate quantities of NO are associated with various pathological conditions of the inner ear. In kidney and gut, the toxicity of cisplatin is markedly reduced by a NOS inhibitor.[1] We have reported that cisplatin induces iNOS expression in the cochlea and an NOS inhibitor reduces the functional disturbance of cochlea.[6] The application of cisplatin causes the apoptosis in the tissue. This phenomenon could lead the functional disturbance. However, details of the mechanism are not yet clarified. DNA fragmentation is a characteristic feature of apoptosis. Fragmented DNA is detected as single-stranded DNA (ssDNA) in the apoptotic cells. The purpose of our study was to examine whether the blockade of NOS activity would lead to a decrease of the apoptotic change in the cochlea after the cisplatin application by means of immunohistochemistry.

Materials and methods

Twenty-four guinea pigs weighing between 250 and 350 g were used in this study. All animals were confirmed to have a positive Preyer's reflex and were examined to be free from otitis media with an operational microscope. Animals were anesthetized adequately with 5% (w/v) ketaminhydrochloride (50 mg/kg body weight) and 2% (w/v) xylazinhy-

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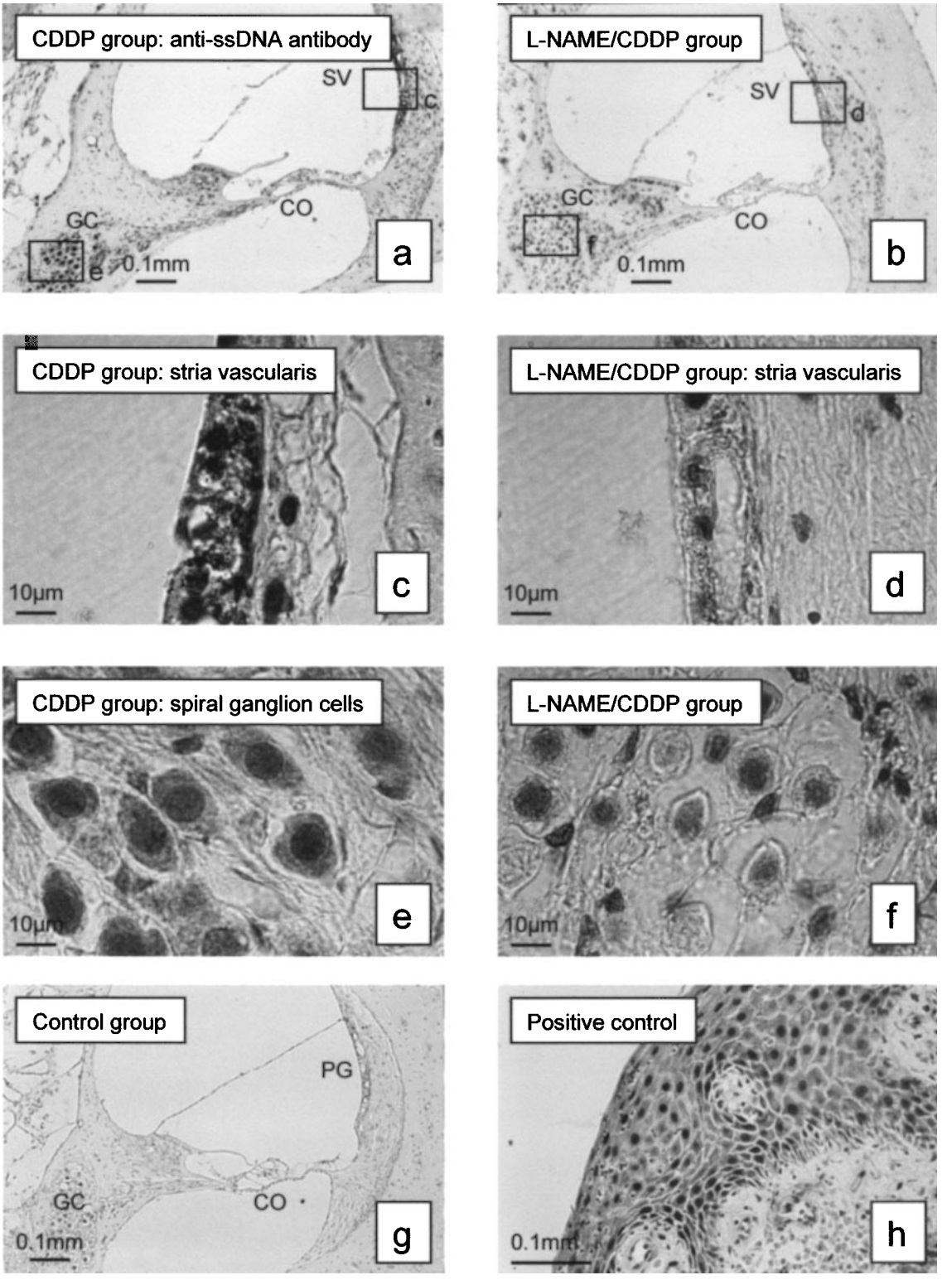

Figure 1. Paraffin sections of the cochlea, 8 μm. Immunohistochemistry, anti-ssDNA antibody. (a) Cisplatin (CDDP)-treated cochlea, anti-ssDNA antibody. ssDNA is mainly detected in the stria vascularis (SV) and the spiral ganglion cells (GC). These immunoreactivities were observed in all turns of cochlea. The organ of Corti (CO) showed no apparent ssDNA immunoreactivity, $\times$15. (b) L-NAME/cisplatin-treated cochlea, anti-ssDNA antibody. Immunoreactivity to ssDNA was similar to that in the cisplatin-treated group; however, the number of cells which exhibited the positive staining for ssDNA was reduced, $\times$15. (c) Cisplatin-treated cochlea, anti-ssDNA antibody. The stria vascularis showed a strong staining for ssDNA,

drochloride (10 mg/kg body weight) before all procedures. The animals were divided into four groups: (i) cisplatin, (ii) NOS inhibitor [N^G-nitro-L-arg-inine methyl ester (L-NAME)] + cisplatin (L- NAME/cisplatin), (iii) L-NAME and (iv) control (NaCl 0.9% w/v).

In the cisplatin group (n=6), 10 mg/kg body weight of cisplatin (0.5 mg/ml, Bristol-Myers Squibb, Tokyo, Japan) dissolved in physiological saline (NaCl 0.9% w/v) was injected i.p. In the L-NAME/cisplatin group (n=6), 50 mg/kg body weight of L-NAME (5 mg/ml; Sigma, St Louis, MO) dissolved in physiological saline (NaCl 0.9% w/v) was injected 1 h before the injection of 10 mg/kg body weight cisplatin. In the L-NAME group (n=6), 50 mg/kg body weight of L-NAME dissolved in physiological saline (NaCl 0.9% w/v) was injected. In the control group (n=6), only physiological saline (10 ml/kg, NaCl 0.9% w/v) was injected. This investigation was approved by the Ethical Committee of Animal Experimentation in Nippon Medical School.

Immunohistochemical examination

All animals were sacrificed 3 days after the injection of each solution. The tissues were fixed via cardiac perfusion with 4% (w/v) 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% (w/v) EDTA solution in Tris (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 (Yamato-koki, Tokyo, Japan). The paraffin was removed by immersion in a graded series of ethanol. Then the sections were immersed in 5% (v/v) H_2O_2 for 30 min, followed by 0.25% (v/v) Triton X for 10 min. Subsequently they were incubated with the first antibody to ssDNA at 1:800 dilution (rabbit polyclonal antibody; A4506, lot no. 0A003A; Dako, Glostrup, Denmark) overnight. After rinsing with 0.1% (w/v) Tris–PBS solution (pH 7.4) and treatment with 3% (v/v) normal goat serum, the sections were incubated with the second antibody at 1:400 dilution (anti-rabbit; Dako) for accentuation. The reaction was developed with a horseradish peroxidase (HRP) complex at 1:100 dilution for 1 h and a nickel-enhanced DAB (Wako, Osaka, Japan).

Results

Immunohistochemical expression of ssDNA

The cochleas in the control group did not show immunoreactivity for ssDNA (Figure 1g). The tissues of squamous cell carcinoma exhibited a positive staining (Figure 1h). In the cisplatin group, the immunoreactivity for ssDNA was detected after 3 days in the lateral wall and spiral ganglion cells (Figure 1a). These ssDNA immunoreactivities were observed in all turns of cochlea. The organ of Corti showed no ssDNA immunoreactivity. In the lateral wall, the stria vascularis exhibited ssDNA expression (Figure 1c). In the modiolus, the spiral ganglion cells showed ssDNA reactivities (Figure 1e). In the basal turn, the morphological change of the organ of Corti was apparent. The degeneration of supporting cells and loss of outer hair cells was observed. The cochleas in the L-NAME cisplatin group showed weak immunoreactivity to ssDNA (Figure 1b, d and f). Comparing the L-NAME/cisplatin group to the cisplatin group, the number of cells in which ssDNA was detected was apparently reduced.

Discussion

The ototoxicity of cisplatin is a well-known side effect. The hearing loss caused by cisplatin in patients is usually bilateral and dominant at high frequencies and progresses to lower frequencies.[8] In animal models, the hearing disturbance also starts from higher frequencies.[8–10] We have shown that the threshold shift of ABR was observed at high frequencies 3 days after the injection of cisplatin in our model.[6]

Cisplatin reacts with DNA and exerts its cytotoxicity by inducing rapid intra-strand cross-linking.[11] Tumor cells which proliferate uncontrollably are damaged by this cytotoxic effect. Recently, another pathway, which involves free radicals, for cytotoxicity of cisplatin is reported.[1,2,6,11,12] In the cochlea, free

$\times$150. (d) L-NAME/cisplatin-treated cochlea, anti-ssDNA antibody. The stria vascularis is shown. ssDNA was detected in some cells of the stria vascularis; however, the number of ssDNA-positive cells was decreased, $\times$150. (e) Cisplatin-treated cochlea, anti-ssDNA antibody. The spiral ganglion cells have an immunoreactivity to ssDNA, $\times$150. (f) L-NAME/cisplatin-treated cochlea, anti-ssDNA antibody. The spiral ganglion cells in the L-NAME/cisplatin group showed weak immunoreactivity to ssDNA, $\times$150. (g) Control group. The natural pigmentation of the stria vascularis was observed (PG); however, the expression of ssDNA was not detected, $\times$15. (h) Positive control, anti-ssDNA antibody. The tissue of squamous cell carcinoma showed a strong staining to ssDNA, $\times$30.

radicals, which have the cytotoxic property, are detected after cisplatin application.[12] In the kidney, cisplatin elevates iNOS activity,[1] and induces the generation of free radicals and subsequent lipid peroxidation.[13] NO is catalyzed from L-arginine.[14] High amounts of NO are catalyzed by iNOS and react with free radicals. Ranjan *et al.*[12] reported that macrophages treated with cisplatin and cytokine produced larger amounts of NO than that with only cisplatin or cytokine. It is supposed that a part of the cytotoxicity of cisplatin is due to the free radicals via the NO pathway mediated by NOS. On the basis of previous reports, we showed that cisplatin induces the expression of iNOS in the cochlea and L-NAME, which is a competitive inhibitor to NOS, reduced the hearing disturbance.[6] This result suggests that the iNOS catalyzed large amounts of NO are partly responsible for the ototoxicity of cisplatin. However, the biochemical details for the ototoxicity of cisplatin in the cochlea is still obscure.

Programmed cell death has a key role during embryogenesis. DNA-damaging drugs are known to induce the apoptosis.[15] In the apoptotic cells, dsDNA is broken to ssDNA by endogenous endonucleases during the process of cell death. The TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method was established to identify the apoptotic cells. dUTP combines to the terminal portion of 3′-OH. However, TUNEL stains not only the apoptotic cells, but also necrotic cells.[16] The sensitivity of TUNEL is limited, because the TUNEL method detects only the late phase of apoptosis accompanied by internucleosomal DNA degradation.[17] The anti-ssDNA antibody reacts to ssDNA chains with more than five or six nucleotides. As compared with the TUNEL method, anti-ssDNA antibodies are more specific and sensitive to apoptotic cells.[18] The immunohistochemical method involving ssDNA is simpler and cheaper than the TUNEL method. The experimental equipment for the immunohistochemistry could be diverted to staining for ssDNA.

Our immunohistochemical results showed that ssDNA was detected in the cochlea after cisplatin administration, particularly in the stria vascularis and spiral ganglion cells. Alan *et al.*[19] reported that the stria vascularis and spiral ganglion cells exhibited a positive reaction to the TUNEL method. Our result supports their observation. Komune *et al.*[9] and Laurell *et al.*[20] reported that the endocochlear potential (EP), which is generated from stria vascularis, was decreased after a single i.v. injection of cisplatin. Cisplatin disturbs the tissue of the stria vascularis and thus the function of the stria vascularis could be lost. Our previous report indicated that iNOS is expressed in the stria vascularis, spiral ganglion cells and the supporting cells of the organ of Corti after the application of cisplatin.[7] NO mediates the neurotoxicity of glutamate through the activation of excitatory amino acid receptors, especially *N*-methyl-D-aspartate (NMDA) receptors.[21,22] Large amounts of NO increase the negative feedback mechanism on the NMDA receptors and might lead the apoptosis in the spiral ganglion cells. L-NAME reduced the apoptotic change in the cochlea. Thus, the ototoxicity of cisplatin is partly mediated by the iNOS catalyzed NO pathway.

Morphological studies indicate that cisplatin destroys outer hair cells in the basal turn.[8,9] iNOS was detected in the supporting cells, but not in the outer hair cells.[6] The degeneration of supporting cells precedes OHC damage[23] leading to morphological and functional depletion. Cisplatin influences the stria vascularis, which is the main energy source of the cochlea. Then cisplatin might indirectly disturb the organ of Corti. The higher metabolic rate of OHC is one reason for the vulnerability to the ototoxicity of cisplatin.[24]

Conclusion

ssDNA was detected in the organ of Corti, the stria vascularis and the spiral ganglion cells of the cisplatin and L-NAME/cisplatin group. Our results suggest, in accordance with immunohistological observations, that NOS inhibitors reduced the cisplatin-induced apoptotic changes and inadequate amounts of NO contribute to the ototoxic effect of cisplatin. ssDNA could be a good parameter for apoptosis.

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