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A MECHANISM OF CISPLATIN ACTION : ANTINEOPLASTIC EFFECT THROUGH INHIBITION OF NEOVASCULARIZATION

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INDEXING WORDS

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SYNOPSIS

Though cisplatin (cis-diamminedichloroplatinum ; CDDP) has been widely used for the treatment of malignant tumors, the mechanism of its action has not been well understood. Neovascularization, which accompanies tumor growth and metastasis, is required for cell proliferation in order to supply both oxygen and nutrients. We have studied in this investigation the effect of CDDP on endothelial cell (EC) proliferation in vitro and on rabbit corneal neovascularization in vivo. DNA synthesis of human umbilical EC was inhibited by CDDP in a dose-dependent fashion. Significant inhibition was observed at concentration over 10^{-8}M, which is attainable in the serum of treated patients. Rabbit corneal neovascularization in vivo was also suppressed by intravenous injection of 0.5mg/kg of CDDP for 10 days. These results suggest that CDDP might have an indirect anti-neoplastic effect through the suppression of neovascularization required for the tumor growth.

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INTRODUCTION

CDDP is an antineoplastic agent made from a platinum complex with a 300 molecular weight. It was discovered to inhibit the growth of Escherichia Coli by B. Rosenberg in 1965. [20] Further investigations of platinum compounds led to the newly developed anticancer drug CDDP in 1969. [21]

CDDP has proven to be an effective antineoplastic agent for the treatment of malignant tumors. It has been reported that CDDP has been successfully used for the treatment of malignant tumors including testicular and ovarian tumors.[9,26] The mechanism of antineoplastic action of CDDP has been described as binding of the incorporated CDDP to the intracellular DNA causing blockage of DNA duplication in the cell. The inhibition of malignant cell proliferation induced by the CDDP has been reported to yield an antineoplastic effect clinically.[27]

Folkman has reported that neovascularization is required for cell proliferation, including malignant tumors, in order to supply both oxygen and nutrients to these cells.[3,4]

It is conceivable that CDDP may effect tumor angiogenesis and indirectly inhibit tumor growth by depriving the cancer cells of the required metabolic support provided from the blood stream. In order to investigate the potential antiangiogenic effect of CDDP, the inhibition of EC proliferation in vitro and neovascularization in vivo was studied.

MATERIALS AND METHODS

I. Preparation of human EC and the measurement of DNA synthesis

ECs were isolated and cultured from human umbilical veins according to the method of Jaffe et al. [11] with some modifications. After the human umbilical vein was treated with 1% solution of collagenase (Worthington, Freehold, N.J.) for 15 minutes at room temperature, the harvested ECs were cultured using RPMI-1640 (Gibco laboratories, Grand Island, N.Y.) supplemented with 50μg/ml of endothelial cell growth supplement (ECGS, Collaborative Research Inc., Lexington, MA.) and 10% of fetal calf serum (FCS, Gibco laboratories, Grand

Island, N.Y.). In the third or fourth passage ECs monolayer culture were used for the following experiments. The rate of DNA synthesis was determined by measuring the uptake of ^{3}H-thymidine (^{3}H-TdR, New England Nuclear, Boston, MA.) into the EC.

ECs were seeded into each well of a 96 multiwell plate (Corning Glass Works, NY) at a cell density of 2 x10^{4}cells/well. The cells were then cultured for 48 hours in the presence of various concentrations of CDDP. Fifteen hours before the end of the culture, 37kBq/well of ^{3}H-TdR was added to the cultures.

At the conclusion of the culture period, the ECs were collected by using 0.25% trypsin (Institute of Microbiology of Osaka University, Osaka, Japan), and the radioactivity of ^{3}H-TdR incorporated into the cells was measured with a liquid scintillation counter.

II. CDDP anti-angiogenic action at the rabbit cornea

In order to investigate the potential anti-angiogenic action of CDDP, the neovascularization model in vivo was developed upon the method of Gimbrone and others [7] in which a micro pocket was created in the corneal stroma of a rabbit.

1 x 10^{6} tumor cells harvested from human uterine cervical squamous cell carcinoma were transplanted to the pocket prepared in the rabbits cornea. A neovascular response was observed from the corneal-scleral junction due to the growth of the implanted malignant cells.

After each rabbit received an intravenous dosage of 0.5 mg/kg of CDDP daily for 10 days, the rabbits were sacrificed and immediately perfused with colloidal carbon fixative. [6] The response of the neovascularization which was observed in the rabbits' cornea administered CDDP was compared with a control group of rabbits to which CDDP was not administered.

The anti-angiogenic effect of CDDP was also investigated in the neovascularization, which was produced by ECGS implanted into the rabbit cornea directly. Hydroxymethylmethacrylate monomer (Polyscience, Warrington, MA) soluted in 100% ethanol was mixed with 5mg of ECGS in order to produce a pellet of the polymer (1.0 x1.0 x 0.5mm) combined with ECGS. Two or three pellets were implanted into the rabbit corneal stroma approximately 2mm away from the

corneal-scleral junction. A neovascular response from the corneal-scleral junction to the embedded pellet was generally observed within 10 days. Histologically this neovascularization was not accompanied by an infiltration of inflammatory cells, which might have indicated that this neovascularization was induced by a direct effect of ECGS, and not from irritation from the pellet.[13]

These rabbits also received an intravenous dosage of 0.5mg/kg of CDDP daily for 10 days. At the conclusion of the 10 day period of intravenous CDDP injection, the rabbits were sacrificed and immediately perfused with colloidal carbon fixative.[6] The response of the neovascularization to the CDDP was compared with that of control rabbits which had received pellet implantation without CDDP administration.

RESULTS

I. The effect of CDDP on human EC DNA synthesis

The incorporation of ^{3}H-TdR into ECs was suppressed by the addition of CDDP with or without ECGS. This inhibition of DNA synthesis by CDDP was observed in a dose-dependent fashion (Fig. 1) . Significant inhibition was observed at concentration over 10^{-8}M CDDP. This concentration of CDDP was identical to that of serum in patients who were treated with CDDP in clinical antineoplastic therapy. Over 90% of the ECs were viable at 10^{-8} M CDDP when examined by trypan blue exclusion. These results suggested that CDDP inhibited cell proliferation not by a cytotoxic effect but through suppression of DNA synthesis.

CDDP inhibited cell proliferation in both the groups treated with and without ECGS. It is, therefore, suggested that CDDP did not modulate ECGS binding to ECs but directly caused the suppression of DNA duplication.

II. Time-kinetic study of CDDP anti-proliferative effect against the EC

The authors performed the following experiment to determine the time-dependent anti-proliferative effect of CDDP on ECs. At various time intervals after starting the EC culture, CDDP was added at a final concentration of 5×10^{-6}M and

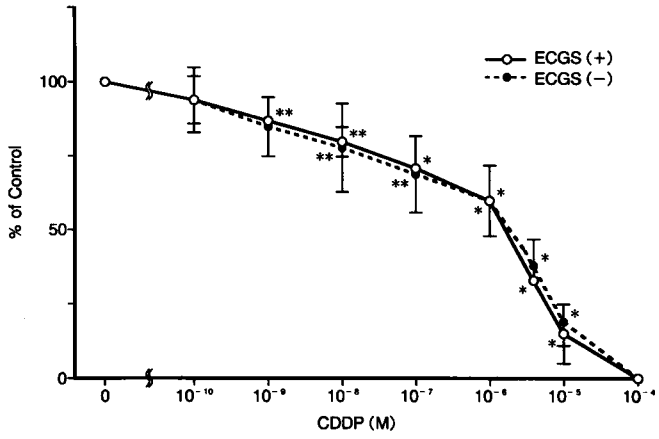

Fig.1. Inhibitory effect of CDDP on DNA synthesis of EC.
ECs were cultured with various concentrations of CDDP in the presence or absence of 50μg/ml ECGS for 48 hours. The rate of DNA synthesis was evaluated by measuring the amount of ^{3}H-TdR incorporation as mentioned in the material and methods. The incorporation of ^{3}H-TdR into ECs was suppressed by the addition of CDDP in the dose-dependent fashion. Significant inhibition was observed at concentration over 10^{-8}M CDDP. Each point and bar represents the mean and SD of 5 separate experiments, each done in triplicate. Experimental values were compared with controls by Student's t test. (*P<0.01,**P<0.05)

the influence of the duration of exposure of CDDP to the ECs was evaluated.

Significant inhibition of DNA synthesis was observed when CDDP was added at the time intervals longer than 30 hours before the end of the assay (Fig. 2). These results showed that the inhibition of DNA synthesis does not occur immediately with exposure to CDDP, but is dependent on adequate exposure over time. This might indicate that retention of CDDP was required for the anti-proliferative effect of ECs through a mechanism that has been previously described.[16)]

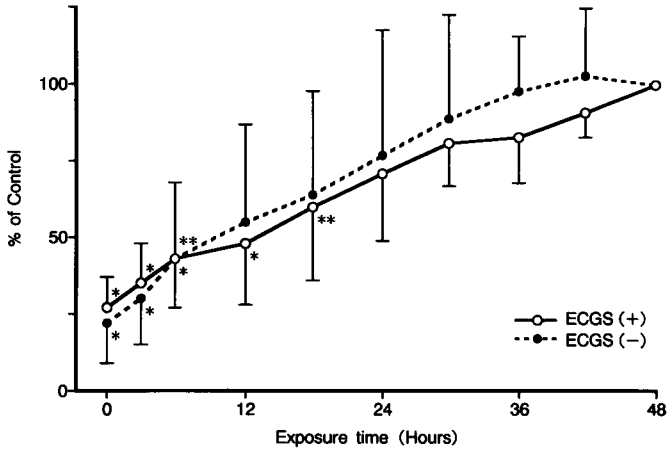

Fig.2. Time kinetic study of CDDP induced suppression of endothelial cell proliferation.
ECs were cultured in the presence or absence of 50μg/ml ECGS. At various time after starting the EC culture, CDDP was added at a final concentration of 5x 10^{-6}M. Significant inhibition of DNA synthesis was observed when CDDP was added at the time intervals longer than 30 hours before the end of the assay. Each point and bar represents the mean and SD of 3 separate experiments, each done in triplicate. Experimental values were compared with controls by Student's t test. (*P<0.01,**P<0.05)

III. CDDP inhibition of neovascularization in the rabbit cornea

Squamous cell carcinoma cells were implanted into the corneal stroma of rabbits. The implantation of these cells resulted in a significant neovascular response from the corneal-scleral junction. This observation suggested that the squamous carcinoma cells might produce angiogenic factors.

The neovascularization was clearly inhibited by intravenous injection of 0.5mg/kg of CDDP for 10 days, (Fig. 3a,3b). CDDP was, therefore, responsible for the inhibition of the neovascular response surrounding the implanted tumor cells.

Intravenous administration of 0.5mg/kg of CDDP for 10 days to rabbits with the implanted ECGS pellet showed inhibition of the neovascularization induced by

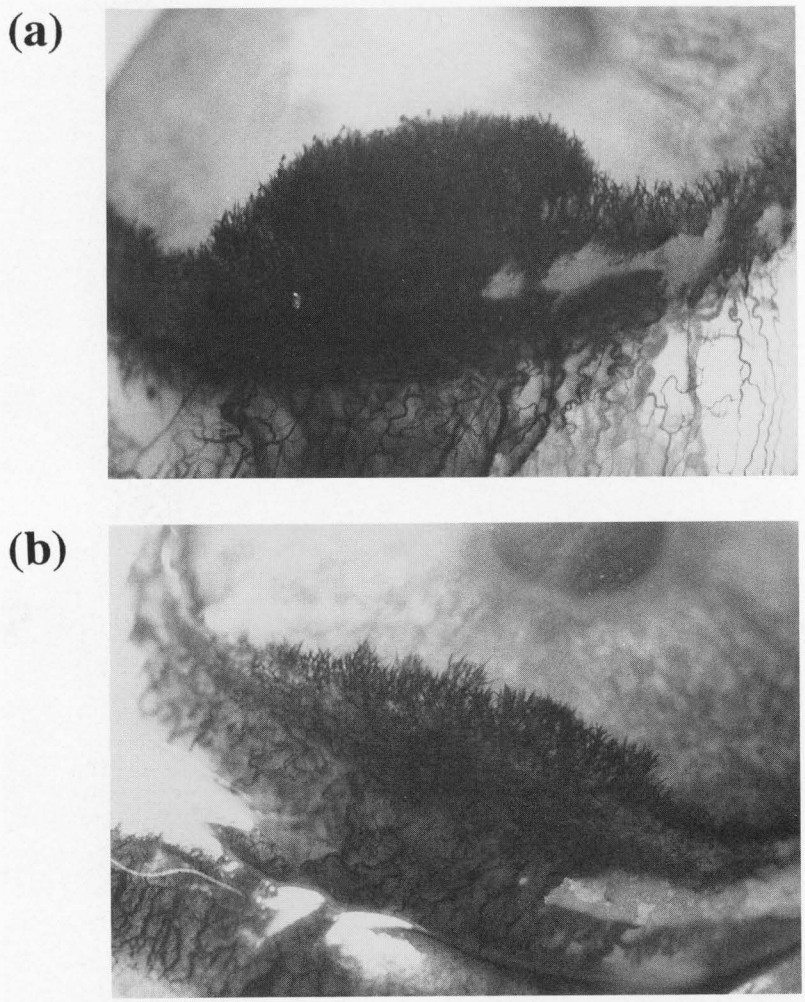

Fig. 3. Effect of CDDP on tumor angiogenesis observed in the rabbit corneal stroma implanted with squamous cell carcinoma.

(a) Corneal neovascularization was observed ten days after implantation of tumor cells.

(b) After implantation of tumor cells, 0.5mg/kg of CDDP was injected intravenously daily for 10 days. Corneal neovascularization was inhibited compared with that of control cornea.

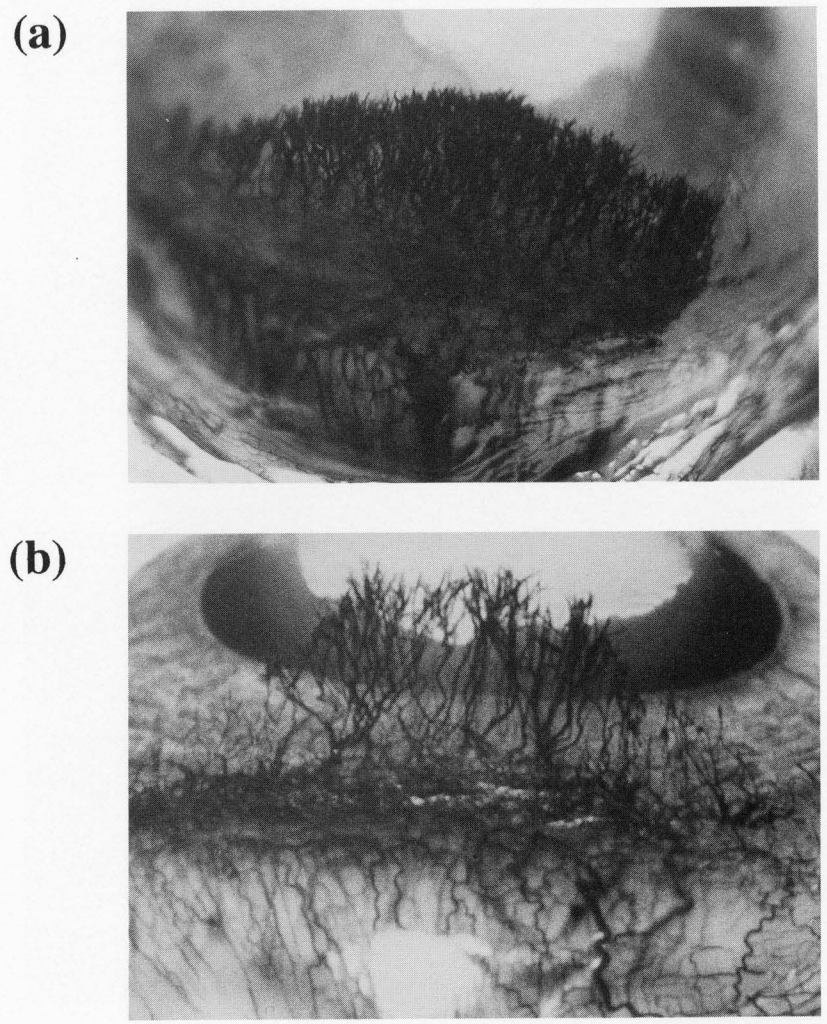

Fig.4. Effect of CDDP on ECGS-induced corneal neovascularization. A hydroxymetylmethacrylate pellet containing ECGS was implanted in the corneal stroma of a rabbit.

(a) Ten days after the implantation of the pellet corneal neovascularization without inflammatory cell proliferation was observed

(b) After implantation of the pellet, 0.5mg/kg of CDDP was injected intravenously daily for 10 days.Corneal neovascularization was inhibited compared with that of control cornea.

ECGS. In these rabbits the anti-neovascular mechanism of action of the CDDP could only be a direct effect against the ECs (Fig. 4a, 4b).

It is thus, suggested that CDDP could inhibit neovascularization by a direct action on the ECs in addition to the indirect effect on implanted tumor cells in the rabbit cornea.

DISCUSSION

The word angiogenesis was initially used to describe the process of neogenesis of blood capillaries at the placenta.[8] However, the term angiogenesis has been widely applied to any newly proliferating vessels that develop a vasoganglion.

Angiogenesis is generally observed during an embryological or primitive growth phase of an organism. Examples of normal angiogenesis include epiphyesal growth, endometrial proliferation, ovulation or leutinization.

Angiogenesis is also observed as a normal component of healing after trauma. However, angiogenesis is also associated with pathological processes. It is a critical component of the growth of solid tumors and the spread and survival of metastatic lesions.[3,19]

The primary event which precipitates angiogenesis is the activation of ECs. The activated ECs produce reactive oxygen species and proteolytic enzymes such as collagenase and plasminogen activator. These substances destroy the basement membrane of the blood vessels permitting angiogenesis to proceed.

After the destruction of the basement membrane, the ECs begin to migrate away from the parent vessel. Simultaneously these ECs begin to proliferate. When the population of new and migrating cells has reached a critical size, lumen formation and lumen anastomosis occurs. This is the first phase in the development of a vasoganglion.

Several kinds of humoral angiogenic factors were reported to stimulate, promote, and control the development and maturation of angiogenesis. Folkman and others were the first scientists to isolate the angiogenic factors from tumor cells. They called these factors "tumor angiogenic factors"of TAFs.[5]

Recently various kinds of growth factors have been identified. These factors include: basic fibroblast growth factor (bFGF), vascular endothelial cell growth

factor (VFGF), and various cytokines. The cytokine TNF-α has been reported to have strong angiogenic activity.[2,14,18,25]

Though it has been reported that monocytes and mast cells might be related to the angiogenesis initiated by tumor cells,[23] angiogenesis is the vital process for the proliferation and survival of solid tumors. It is recognized that if tumor mediated angiogenesis could be halted, this would provide very powerful control over the enlargement, proliferation, and spread of tumors. Many kinds of angiocidal or angiostatic substances have been identified. These include angiostatic steroids,[1] sulfated chitin derivatives,[17] fumagillin analogue (TNP-470),[10,12] platelet factor-4,[15] γ-interferon,[22] and protamine.[24]

In this study it was clearly shown that physiological attainable concentrations of CDDP inhibited DNA synthesis of ECs in vitro. It was also shown that the action of CDDP against EC DNA synthesis was potentiated by an adequate period of exposure. The greater the amount of time during which CDDP had to enter a cell and interact with the nuclear DNA, the better the inhibition of DNA synthesis.

It is very probable that the mechanism of action of CDDP against nuclear DNA synthesis is the same for both ECs and tumor cells. The blockage of DNA synthesis effectively inhibits cell replication and inhibits tumor growth. An indirect benefit of inhibition of tumor growth would be the parallel inhibition of angiogenic growth factors which might have come from unrestrained tumor growth.

CDDP in vivo was clearly shown to block squamous cell carcinoma induced angiogenesis at the rabbit's corneal stroma by inhibiting the EC proliferation directly. This is because CDDP was shown to directly block angiogenesis that had developed by the implantation of pellets containing ECGS in vivo.

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