The Antiviral Agent Cidofovir [(S)-1-(3-Hydroxy-2-phosphonylmethoxypropyl) Cytosine] Has Pronounced Activity against Nasopharyngeal Carcinoma Grown in Nude Mice

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Abstract

The effect of the antiviral agent (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (cidofovir) on the EBV-associated tumor nasopharyngeal carcinoma (NPC) was evaluated in NPC xenografts in athymic mice. Intratumoral injection arrested tumor growth within 1 week, and by 4 weeks, tumors regressed to 8–75% (39 ± 33%) of the original size, whereas control tumors injected with PBS grew to 282 ± 25% of the original size. Ganciclovir slowed but did not arrest or cause regression of tumor growth. A striking antitumor effect was also produced by systemic administration; at 4 weeks, tumors were 79 ± 49% of the original size, compared with 635 ± 91% for the controls. Widespread apoptosis was detected after treatment for 2–6 days in C15 as well as two other NPC xenografts, C17 and C18; the latter NPCs have mutations in the p53 gene. These data indicate that cidofovir induces rapid cell death through apoptosis in EBV-transformed epithelial cells.

Introduction

In recent years, the potent and selective anti-DNA virus activity of cidofovir has been discovered and characterized. Cidofovir belongs to a new class of antiviral molecules, the acyclic nucleoside phosphonate analogues. These molecules are active in vitro and in vivo against a broad range of DNA viruses including herpesviruses, poxviruses, papillomaviruses, polyomaviruses, papillomaviruses, and adenoviruses (1). This class of molecules is characterized by a stable phosphonate linkage between the acyclic nucleoside and the phosphate moiety. As a consequence, and in contrast to antiviral drugs such as acyclovir, penciclovir, and ganciclovir, cidofovir bypasses the first phosphorylation step by herpesvirus-encoded kinases. On further phosphorylation by cellular kinases, the diphosphorylated metabolite selectively inhibits the viral DNA polymerization process. Hence, the compound has potent activity against thymidine kinase-deficient herpesvirus strains and cytomegalovirus strains that are deficient in their ability to phosphorylate ganciclovir. Cidofovir has shown promise in clinical trials for herpetic infections (2) as well as ocular adenovirus infections (3) and has recently been approved for the i.v. treatment of cytomegalovirus retinitis in patients with AIDS (4). Cidofovir is a potent inhibitor of the replication of EBV in cell culture (5). Inhibition of EBV replication was confirmed by the finding that a single i.v. administration of 5 mg/kg cidofovir produced complete regression of oral HLP (6), which is characterized by abundant EBV replication (6). The major side effect of cidofovir is nephrotoxicity. However, combined use with probenecid reverses this adverse effect without affecting the antiviral potency of the compound (4). Side effects have not been observed on topical application of cidofovir (2).

In addition, cidofovir produces complete regression of Shope papilloma virus-induced lesions in rabbits. Subsequently, a similar dramatic effect of cidofovir was observed in several patients with severe recurrent laryngeal papillomatosis caused by HPV (7). Cidofovir also proved highly effective in the topirical treatment of anogenital HPV infections in AIDS patients with severe, relapsing penile, perigenital, intra-anal, or cervical and vulvar condylomata associated with HPV-16 (8). More extensive clinical trials are in progress to determine the safety and efficacy of cidofovir against HPV-associated lesions.

NPC, which is universally associated with EBV, is endemic in Chinese in Southeast Asia. In southern China, NPC may account for 20% of all cancers (9, 10). An age-adjusted incidence of 26 cases per 100,000 has been reported among males in Hong Kong. Although resection of the tumor, together with radiation therapy and/or chemotheraphy (cisplatinum and 5-FU), has proven to be effective, relapse and metastasis occur frequently (11, 12).

Because NPC, like HPV papillomatisis, is a tumor of epithelial origin induced by an oncogenic DNA virus, EBV, it was thought that NPC might respond to the compound. However, in contrast to HLP, in which there is active replication of EBV, in NPC, EBV infection is mainly latent, and the episomal DNA is replicated by a host DNA polymerase (9). Importantly, in both HPV papillomatisis and NPC, the viral episomal forms are both transcribed and replicated by host polymerases. In this study, cidofovir is shown to have potent activity against NPCs that were grown in athymic mice.

Materials and Methods

Nasopharyngeal Tumors. NPC C15 was established from the primary nasopharyngeal tumor of a 13-year-old girl; NPC C17 and NPC C18 were established after radiation and chemotherapy from recurrent cutaneous and cervical lymph node metastases, respectively (13). The tumors were propagated by s.c. passage in athymic nude mice. Tumors (1–2 cm³) were dissected and trimmed into 2-mm³ pieces. After a brief rinsing in PBS, one-third or one-fourth of each tumor was mixed with Matrigel and transplanted s.c. in the mice. Tumors appeared in about 70% of the animals 7–8 weeks posttransplantation.

Treatment and Evaluation. For intratumoral treatment, tumors between 0.5 and 1.5 cm³ were injected with 100 µl of a 1.5% solution of cidofovir (Gilead Sciences, Foster City, CA) in PBS that was neutralized to pH 7.4 and sterilized through filtration (Millex-GS, 0.22 µm; Millipore). Control animals received PBS only. For systemic treatment, animals were injected s.c. in the
Cidofovir and NPC

Cytostatic activity in cell culture comparable to or greater than that of cidofovir is another nucleoside analogue with anti-EBV activity that has shown similar antitumor effects in a mouse model. The tumors were dissected at different time points after the start of treatment, and portions of the tumors were fixed in 10% buffered formaldehyde and embedded. Paraffin sections were stained with H&E. In situ hybridization for EBER RNA was performed as described previously (10, 14) using a fluorescein-tagged 30-bp oligonucleotide complementary to the EBER1 RNA sequence from nucleotides 6697–6726. After deparaffinization, sections were digested for 10 min with 25 μg/ml proteinase K. Prehybridization was performed with a solution containing 20 mM sodium phosphate. Denhardt’s solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA) for 30 min at 37°C followed by a 2-h hybridization in the prehybridization solution containing 50 nmol/ml labeled oligonucleotide at 37°C. Hybridization was detected by incubation with rabbit antifluorescein antibody tagged with alkaline phosphatase. followed by reaction with the substrate, 5-bromo-4-chloro-3-indolyl phosphate, and the colorimetric indicator, nitroblue tetrazolium. Detection of apoptosis in the tumor sections was by the TUNEL test according to the manufacturer’s instructions (Boehringer Mannheim). Sections were evaluated by fluorescence microscopy.

Results

In the first set of experiments, 100 μl of cidofovir (1.5%; 60 mg/kg/day) were injected into C15 tumors that had reached sizes of 0.5–1.5 cm³ (Fig. 1A). Four to 5 days after the start of treatment, white patches appeared on the surface of most of the cidofovir-injected tumors. Overall, tumor size was reduced to 39 ± 33% of the initial tumor volume 4 weeks after the initiation of cidofovir treatment. A second NPC xenograft (C17) also regressed to 87% of the initial tumor volume after intratumoral treatment with cidofovir (data not shown). Control C15 tumors that had been injected with PBS at the same time grew to sizes of 282 ± 25% of the initial volume. Ganciclovir is another nucleoside analogue with antieBV activity that has cytostatic activity in cell culture comparable to or greater than that of cidofovir (15). Although growth of the ganciclovir-treated tumors was reduced as compared to the controls (125 ± 17, 146 ± 11, 154 ± 34, and 157 ± 21% of the initial size at 7, 14, 21, and 28 days, respectively, after start of treatment), in contrast to the cidofovir-treated tumors, regression in tumor size was not observed.

To determine whether systemic treatment with cidofovir was also effective in reducing the growth of the C15 tumors (Fig. 1B), cidofovir (100 mg/kg/day, 5 days a week) was administered s.c. starting with tumors that had reached sizes of 0.25–0.65 cm³. Four weeks after the start of treatment, most of the tumors had not increased in size but had regressed (mean tumor size, 79 ± 69% of the size at the start of treatment). Control tumors increased in the same time span to volumes of 635 ± 91% of the initial size.

To determine the effect of cidofovir on the tumors at early times after the start of treatment, the tumor masses were dissected and processed for histology, in situ hybridization for EBER RNA, and staining for apoptosis. At the start of treatment in the animals treated with PBS, distinct epithelial tumor masses of typical undifferentiated NPC surrounded by mouse stromal tissue were present (Fig. 2A). In these tumors, a small number of apoptotic cells were detected (Fig. 2B). As early as 2 days after intratumoral injection with cidofovir, histological examination revealed loss of cellularity, anuclear cellular debris, vascular congestion and hemorrhage, and infiltrating cells (Fig. 2C). Condensed nuclei in H&E-stained sections suggested an increase in apoptotic cells that was confirmed by TUNEL staining. The apoptotic cells were concentrated in the affected area, in contrast to the small number of apoptotic tumor cells in the unaffected region of the tumor (Fig. 2D). This finding was consistent with the macroscopic appearance of white patches on the tumor surface 4–5 days after the start of cidofovir treatment.

On day 6, matched sections revealed almost complete destruction of tumor with large regions of anuclear debris, apoptotic tumor cells, and large numbers of infiltrating lymphocytes, neutrophils, and macrophages (Fig. 3, A and D). A remnant of viable tumor cells adjacent to the capsule still contained detectable EBERs (Fig. 3, B and E, left) and was TUNEL-negative (Fig. 3, C and F, left). EBER RNAs are abundant, nonpolyadenylated small nRNAs that are present in approximately 10⁶ copies per infected cell. These RNAs are not expressed in HLP and are considered a marker of latently infected, EBV-transformed cells (6). However, the main body of the tumor was massively apoptotic (Fig. 3, C and F, right) and EBER negative (Fig. 3, B and C).
CIDOFOVIR AND NPC

Fig. 2. Effects of cidofovir early after inoculation. Histological sections of the CI5 tumor are shown before the start of treatment (A and B) or 2 days after the start of intratumoral injection of cidofovir (C and D). H&E staining, A and C; TUNEL staining, B and D. Magnification, ×200 (A and B) and ×50 (C and D).

E). By 4 weeks, the remaining tumor consisted almost entirely of amorphous dead tissue.

NPCs carrying a mutant p53 gene, C17 and C18, were also analyzed (16). Histological examination after intratumoral injection with cidofovir for 6 days revealed extensive apoptosis (Fig. 4). The untreated animals had viable tumors with few apoptotic cells (Fig. 4, A, B, E, and F), whereas the treated C17 and C18 tumors had a histological appearance with massive apoptosis, similar to the C15 tumors (Fig. 4, C, D, G, and H).

Discussion

This study was designed to determine whether the antiviral agent cidofovir has potential antitumor activity against NPC. In its diphosphorylated form, cidofovir inhibits herpesvirus-encoded DNA polymerases (17, 18). The compound is a potent inhibitor of the cytolytic replication of EBV (5) but does not affect EBV episomal copy number or the growth of latently infected cells (15). The C15, C17, and C18 tumors have many of the characteristic features of NPC including histology, cell markers, maintenance of a fixed EBV episomal copy number, expression of type 2 EBV latency antigens, and lack of viral replication (13). Therefore, the mechanism of the inhibitory effect of cidofovir on growth of NPC must be distinct from its antiviral polymerase activity, because the C15 as well as C17 and C18 cells, which are latently infected, do not express the EBV DNA polymerase. Although HPV is produced in papillomas, whereas NPC represents a non-virus-producing transformed cell infection state, in both cases the viral genomes are replicated as episomes by host DNA polymerase, and viral genes are expressed by host RNA polymerase. Cidofovir may possibly affect one of these biochemical processes.

In the present study, there was a dramatic decrease in the size of

Fig. 3. Decreased EBV EBER expression during apoptosis induced by cidofovir. Matched sections of C15 tumor sampled on day 6 of treatment were stained with H&E (A and D), hybridized in situ for EBER (B and E), or stained with the TUNEL test (C and F). Magnification, ×100 (A, B, and C) and ×400 (D, E, and F).
established NPCs in mice when cidofovir was injected intratumorally. Systemic treatment with cidofovir also produced a striking tumoristatic effect with substantial tumor regression in half the animals. The observation that not all tumors regressed may reflect the lower concentration of cidofovir in the tumors in animals receiving the compound s.c. Ganciclovir, which has greater cytostatic activity than cidofovir in cell culture, did not cause tumor regression on intratumoral injection; tumors continued to grow, although they grew more slowly.

As early as 2 days after the start of treatment, there was a large increase in the number of apoptotic tumor cells. Drug-induced apoptosis is a mechanism by which many antitumor agents elicit antitumor activity (19). Cidofovir is a compound with low in vitro cytotoxic and cytostatic action (20) (e.g., more than 10,000-fold less cytostatic than cytarabine or daunorubicin). Cidofovir also induces apoptosis in HPV-containing cell lines, paralleling the pronounced antitumor activity of cidofovir against HPV papillomas. Although NPC cannot be

Fig. 4. Induction of apoptosis in NPC without p53 mutations. Matched sections of C17 (A–D) and C18 (E–H) tumors, either untreated (A, B, E, and F) or treated for 6 days (C, D, G, and H), were stained with H&E (A, C, E, and G) and with the TUNEL test (B, D, F, and H). Magnification, ×200.
cultured in vitro for similar studies, the tumor cells seemed to be specifically targeted, because the surrounding tissues were not affected by the compound.

Nucleoside analogues with antitumor activity such as arabinocytosine, gemcitabine, and 5-FU as well as other agents, e.g., daunorubicin, cisplatinum, and etoposide, induce apoptosis. The signaling pathways that initiate drug-induced apoptosis remain largely unknown but are distinct from those associated with apoptosis induced by the FAS receptor (21). In addition, the induction of apoptosis by cidofovir must not be dependent on the p53 gene, because the C17 and C18 tumors have deletions in the p53 gene, whereas C15 has wild-type p53, as do most NPCs (16). Gemcitabine monophosphate induces apoptosis due to incorporation into cellular DNA (22). Interestingly, cidofovir also has the potential to incorporate into DNA (23). It is also possible that disruption of the cell cycle, shown for 5-FU and methotrexate (24), may be a trigger for apoptosis.

Tumor viruses encode proteins that inhibit apoptosis in infected cells. The HPV E6 gene promotes p53 degradation, and EBV also expresses proteins that inhibit apoptosis. In permissive infection, EBV encodes a gene, *BRHF1*, that is homologous to bcl2 and inhibits apoptosis. In latent infection, LMP1 specifically inhibits p53-mediated apoptosis though induction of expression of the bcl2 and A20 genes (25). The absence of detectable EBER RNAs in the affected tissue raises the possibility that there is inhibition of viral expression, which might enhance susceptibility to apoptosis either from inducers such as tumor necrosis factor or possibly through the DNA-damaging effects of cidofovir incorporation.

In the clinical setting, intralesion treatment of HPV papillomas with cidofovir has been well tolerated. Doses used were 1.25 mg/kg or 3–12 ml of a 2.5% cidofovir solution (7). Side effects were not observed with these doses, and most papillomas disappeared completely. Thus, intratumoral treatment of NPC with cidofovir may be expected to be well tolerated. When combined with protrabevic, toleration of systemic treatment with cidofovir is improved (26). Moreover, the cyclic ester derivative of cidofovir, c-HPMPC, is 13-fold less nephrotoxic than the parent compound in animal models; however, c-HPMPC seems to be hepatotoxic (27). Intracellularly, c-HPMPC is readily converted to cidofovir by cyclic CMP phosphodiesterase (28). Because c-HPMPC can be viewed as the intracellular prodrug of cidofovir, it is expected to have anti-NPC activity similar to that of cidofovir and possibly an improved safety profile. The activity of c-HPMPC or other analogues will be the subject of additional studies of treatment of NPC.

The observation that systemic treatment with cidofovir is able to halt NPC growth at distant sites is likely to have additional clinical implications. In patients that are at high risk of relapse and for the development of metastasis, cidofovir, c-HPMPC, or one of the related analogues may be useful for adjunctive treatment. Cidofovir, which also inhibits lytic and transcriptional studies of treatment of NPC, infection, cidofovir, c-HPMPC, or one of the related analogues will be the subject of additional studies of treatment of NPC.

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References

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