In vivo selective and distant killing of cancer cells using adenovirus-mediated decorin gene transfer[1]

J. GUILHERME TRALHÃO, LILIANA SCHAEFER,* MIROSLAVA MICEGOVA,* CESAR EVARISTO, ELKE SCHÖNHERR,† SAMER KAYAL,‡ HENRIQUE VEIGA-FERNANDES,§ CLAIRE DANEL, RENATO V. IOZZO,‖ HANS KRESSE,† AND PATRICIA LEMARCHAND[2]

INSERM E0016, Université René Descartes, Faculté Necker-Enfants Malades, Paris, France; *Department of Internal Medicine, University of Münster, Germany, †Institute of Physiological Chemistry and Pathobiochemistry, University of Münster, Germany; ‡INSERM U411, Faculté Necker-Enfants Malades, Paris, France; §INSERM U345, Faculté Necker-Enfants Malades, Paris, France; and ‖Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA

SPECIFIC AIMS

Decorin is an ubiquitous proteoglycan that is a normal component of the extracellular matrix and has recently been described as capable of retarding the growth of various tumor cells, both in vitro and in vivo. Our aim was to evaluate the potential for cancer gene therapy using adenovirus-mediated gene transfer of human decorin.

PRINCIPAL FINDINGS

1. In vivo distant anti-tumoral effect of adenovirus-mediated decorin gene transfer

We used a xenograft model in which A549 cells (a human lung carcinoma cell line) were inoculated subcutaneously (s.c.) to the right hind flank of nude mice. After establishment of a sizable tumor, mice were injected intratumorally on days 0, 3, 6 with a first generation adenovirus (Ad) vector encoding the human decorin cDNA (Ad.DCN) with a similar Ad vector containing no exogenous gene and used as a control (Ad.null), or with phosphate buffer saline (PBS). No statistical difference was observed between Ad.null and PBS groups at any time. In contrast, a significant reduction in tumor growth was observed in the Ad.DCN group compared with the Ad.null control group ($P<0.003$; **Fig. 1**, graph I).

We then evaluated tail vein injections of Ad vectors, since such administration into the venous circulation target most of the viral particles to the liver without significant intratumoral infection. Growth inhibition of s.c. xenografts was observed similar to growth inhibition secondary to intratumoral Ad injections, demonstrating a decorin antitumoral effect remote from decorin expression site ($P<0.001$; Fig. 1, graph II). To confirm decorin remote antitumoral effects, a xenograft model composed of one s.c. tumor on each hind flank was established. Only one tumor was injected with the Ad vector and both were measured over time. Intratumoral injections of Ad.DCN induced growth inhibition of the injected tumor together with parallel growth inhibition of the contralateral uninjected tumor ($P<0.001$; Fig. 1, graph III). Finally, five repeated intratumoral injections of Ad.DCN vector led, after each injection, to significant reduction in tumor volume similar to the initial one (Fig. 1, graph IV), demonstrating that no short-term resistance against decorin apoptotic effect was induced. Northern blot analysis of liver and tumors from Ad.DCN mice confirmed the local expression of decorin at the injected site and showed an absence of decorin expression at distant sites, e.g., in noninjected tumors. This indicates that distant decorin anti-tumoral effect was not due to Ad vector spreading.

The presence of the decorin protein at the contralateral tumor site or within distant tumors after Ad.DCN i.v. injection was demonstrated using immunochemistry and a monospecific anti-decorin antibody. A positive staining of the extracellular tumor matrix confirmed the mRNA data. Western immunoblotting of the tumors confirmed the presence of decorin in Ad.DCN injected tumors either i.v. or i.t. and in contralateral tumors, but not in controls. Quantitative measurements of decorin levels in whole tumors showed great decorin protein increase over background in i.t. injected tumors, i.v. injected tumors, and a slight though not significant increase in contralateral. Collectively, these results confirmed that decorin could migrate to the distant tumor site. Decorin delivery to the distant tumors was confirmed by induction of endogenous p21, a well-known downstream mediator of decorin effects that is induced upon de novo expression of decorin. Using Northern analysis with a human p21 cDNA probe, we observed p21 overexpression in

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[2] Correspondence: INSERM U533, Faculté de Médecine, 1 rue Gaston Veil, F-44035 Nantes cedex 1, France. E-mail: patricia.lemarchand@nantes.inserm.fr

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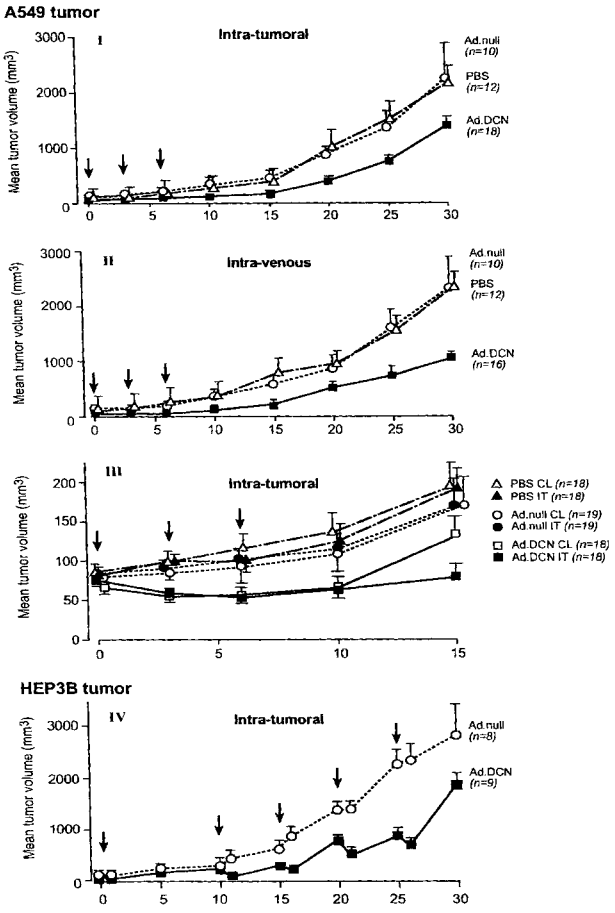

Figure 1. Evidence of a major local and distant antitumoral effect in vivo, using Ad-mediated decorin gene transfer. Nude mice were injected s.c. with human lung A549 or human liver Hep3B xenograft cells. When tumors reached volume >50 mm^3, an Ad vector encoding human decorin cDNA (Ad. DCN), a control vector (Ad.null), or PBS was injected in vivo on days 0, 3, 6 (arrows) intratumorally (graphs I, III, IV) or i.v. (graph II). In graph III, 2 distinct xenografts were concomitantly established (one on each flank); only one tumor was injected with the Ad vector (intratumoral, IT) and the contralateral (CL) tumor remained uninjected.

decorin expression sites, tumors of animals injected with Ad.DCN i.v., or contralateral uninjected tumors of animals injected intratumorally with Ad.DCN.

2. Adenovirus-mediated decorin gene transfer induces tumor cell apoptosis both in vitro and in vivo

Because the reduction in tumor volume after Ad.DCN intratumoral injection was rapid but transient, followed by tumor growth starting again on day 5 after a single Ad.DCN injection, we suspected a rapid extinction of decorin overexpression. Northern analysis of decorin overexpression over time in Ad.DCN injected tumors confirmed this hypothesis, with a 50% decrease in decorin mRNA on day 6 compared with day 2, and a concurrent 47% decrease in p21 mRNA. Thus, we hypothesized that decorin overexpression could directly induce tumor cell apoptosis possibly via an autocrine mechanism. Because of low gene transfer efficiency, persistence of nontransduced cells after apoptosis of decorin overexpressing cells may induce rapid recovery of tumor growth. TUNEL analyses of Ad.DCN injected tumors were performed together with immunochemistry using an antibody against human decorin. Fewer than 1% of the tumor cells were transduced after a single intratumoral injection of Ad.DCN, a very low level of gene transfer efficiency confirmed by experiments using an Ad vector expressing the β-galactosidase reporter gene. TUNEL analyses on subsequent slides revealed tumor cell apoptosis, in large regions surrounding the decorin-positive cells, demonstrating an in vivo paracrine anti-neoplastic effect of decorin and no apoptosis in Ad.null injected tumors. Despite very low gene transfer efficiency, a $\sim 11 \pm 13\%$ decrease in tumor volume was observed on day 3 after a single Ad.DCN injection (vs. $118 \pm 65\%$ increase in Ad.null mice), showing the importance of decorin-induced apoptosis. Finally, apoptosis was observed in tumors from animals injected i.v. with Ad.DCN and in contralateral uninjected tumors in animals injected intratumorally with Ad.DCN, but not in tumors injected i.v. with Ad.null. These results confirm a proapoptotic effect of decorin distally from its expression site. Tumor cell apoptosis was confirmed in vitro with FACS analysis and with quantitation of caspase 8 activity, a prototypic initiator of the death domain receptor pathway. Caspase-8 activity was significantly increased in tumor cells overexpressing DCN.

3. Decorin proapoptotic effects are specific for tumor cells

When decorin was expressed in the liver of mice injected i.v. with Ad.DCN, apoptosis was not observed in normal hepatocytes. One plausible explanation of these data is that human decorin could specifically influence human cells. However, this is less likely since an increase in p21 expression was also observed in the liver of mice overexpressing human decorin, demonstrating that human decorin could up-regulate mouse p21 expression and suggesting that decorin has a specific proapoptotic effect on cancer cells. To further verify this hypothesis, we tested in vitro and in vivo a human hepatocyte cell line, Hep3B, in parallel with in vitro studies of human hepatocytes in primary culture. A dose-dependent growth inhibition was observed in vitro on decorin overexpression in Hep3B cells, and intratumoral Ad.DCN injections in Hep3B xenografts induced an in vivo growth inhibition similar to that observed in A549 xenografts. In marked contrast, when normal human hepatocytes were incubated in vitro with Ad.DCN, there was no reduction in cell survival, indicating that decorin may have a selective anti-tumoral activity.

Because normal hepatocytes are quiescent and not comparable to proliferating tumor cells and to evaluate whether we could take advantage of decorin selective oncolytic property, we analyzed in vitro decorin effects

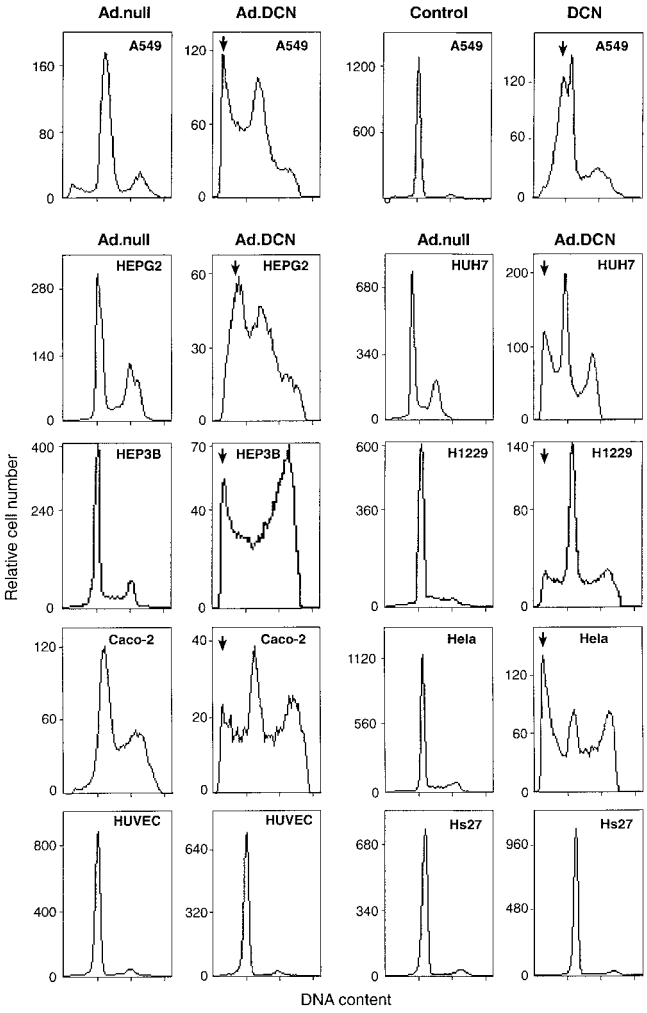

Figure 2. In vitro selective killing of tumoral cells, using Ad-mediated decorin gene transfer. Human lung A549, H1299 tumor cells, liver Hep3B, HUH7, HepG2 tumor cells, colorectal adenocarcinoma Caco-2 cells, cervical adenocarcinoma HeLa tumor cells, human umbilical vein endothelial cells (HUVEC), and human fibroblasts (Hs27) were incubated in vitro with an Ad vector encoding human decorin cDNA (Ad.DCN) or a control vector (Ad.null). FACS analysis of cell cycle 6 days after in vitro incubation with Ad vectors. The arrows indicate a peak of cells in sub-G_1 phase. The top right panel shows A549 cells incubated with 300 μg/mL purified decorin.

on A549 human tumor cells in parallel with primary (nonimmortalized) human proliferating cells: endothelial cells and fibroblasts. Cells were incubated with Ad.DCN and cell proliferation was measured over time. Apoptosis was assessed by analysis of cell cycle using flow cytometry. A549 underwent growth inhibition and apoptosis, as evidenced by the appearance of a 40% sub-G1 peak on day 6 (**Fig. 2**, top left panel) on decorin expression. In contrast, nonimmortalized human cells were highly resistant to decorin, with no inhibition of cell growth and no apoptosis, despite decorin and p21 overexpression. Absence of apoptosis in normal cells was further confirmed by lack of caspase-8 activity. Finally, we evaluated proliferation and apoptosis in several human tumor cell lines with a different genetic background and cellular origin from that of A549 and Hep3B cells. When transfected with Ad.DCN, each tumor cell line demonstrated inhibition of cell proliferation and/or the generation of a sub-G1 peak using FACS analysis (Fig. 2), suggesting that decorin pro-apoptotic effect was independent of a specific genetic background.

CONCLUSIONS

Numerous studies using Ad-mediated gene transfer have been published with direct i.t. injections in various human xenograft models. These studies have focused on transducing tumor cells with tumor suppressor genes or genes encoding proteins associated with the cell cycle. Decorin has the advantage of being a secreted proteoglycan that can target both the immediate extracellular space and matrix as well tumor cells themselves; the latter target could also be reached distally from the primary tumor site. Regardless of efficiency in cell killing, the success of restoring apoptotic response in tumor cells depends considerably on the extent to which such therapies confine death to the cancer cells while allowing survival of normal tissue. Many conventional chemotherapies induce significant toxicity, whereas our results demonstrate that overexpression of decorin, a natural ubiquitous proteoglycan, leads to no apoptosis in healthy tissues; in situ injections of Ad.DCN into an established tumor can result in significant, though temporary, local and distant growth suppression. These results suggest the potential for decorin gene therapy as a novel therapeutic modality for treatment of primary and metastatic cancers. FJ

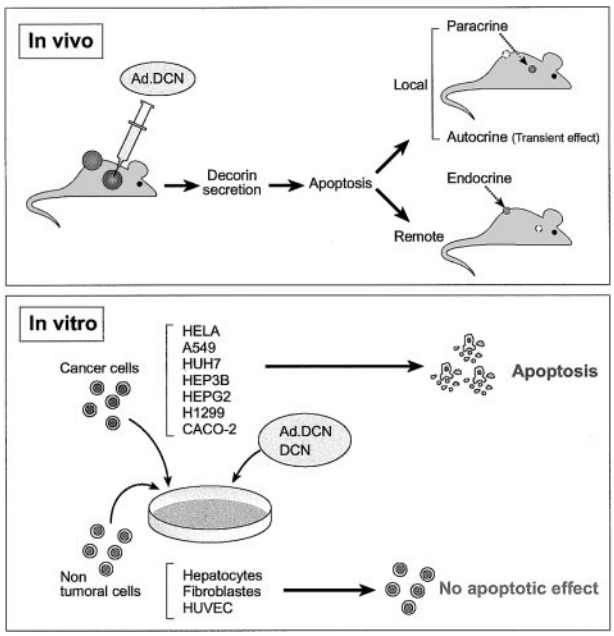

Figure 3. Schematic illustration of the findings.