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Gene therapy with adenoviral plasmids or naked DNA of VEGF and PDGF accelerates healing of duodenal ulcer in rats

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AV:	Adenoviral vector
GFP:	green fluorescent protein
i.d.:	intraduodenal
i.v.:	intravenous
ND:	naked DNA
PAS:	periodic acid-Schiff

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ABSTRACT

After we demonstrated that daily intragastric administration of angiogenic growth factors like basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), or vascular endothelial growth factor (VEGF) accelerated the healing of chronic duodenal ulcers in rats, we hypothesized that a single dose of gene therapy related to these growth factors may be enough to accelerate the healing of duodenal ulcers through enhancement of synthesis of endogenous angiogenic growth factors. Thus, we compared the effects of intraduodenal or intravenous adenoviral vectors and naked DNA transducing the genes for either VEGF or PDGF in experimental duodenal ulcers induced by cysteamine in rats. Sprague-Dawley female rats with confirmed duodenal ulcers were randomly divided into control and treatment groups. The controls received either intraduodenal injection of buffer or the ß-galactosidase transducing adenoviral vector. Rats treated with a single or double dose of adenoviral vector or naked DNA of VEGF or PDGF had significantly smaller ulcers than the controls. Histologic analysis demonstrated that reepithelized granulation tissue with prominent angiogenesis replaced the ulcers. Western blotting, immunohistochemistry and ELISA of duodenal mucosa confirmed that the expression of VEGF or PDGF proteins was enhanced by the transgenes, while ß-galactosidase staining in multiple organs identified that the transgenes, especially after local administration were only localized in the duodenum, stomach and jejunum. These results suggest that gene therapy with either VEGF or PDGF may be a rapid approach to achieve duodenal ulcer healing.

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Duodenal ulcer is the most prevalent form of peptic ulcer, e.g., about 2-4 times more frequent than gastric ulcers (Sonnenberg, 1997). Our laboratory developed easily reproducible models of duodenal ulcer in rats by using cysteamine and related chemicals (Szabo and Selye,1972; Selye and Szabo, 1973; Giampaolo et al., 1991). These animal models can be used to study the pathogenesis of duodenal ulcers including the biochemical and molecular mechanisms of duodenal ulceration and potential strategies to promote healing.

Previously we reported that daily intragastric administration of angiogenic growth factors like basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), or vascular endothelial growth factor (VEGF) accelerated the healing of chronic duodenal ulcers in rats (Szabo et al., 1994; Szabo and Sandor, 1996; Szabo et al., 1998). The healing effect of these peptides is 2-7 million times more potent, on molar basis, than that of antisecretory drugs such as histamine-2 receptor antagonists and proton pump inhibitors (Szabo and Sandor, 1996; Szabo et al., 1998; Szabo et al., 1999). The mechanism of action of the growth factors did not involve inhibition of gastric acid or stimulation of mucus-bicarbonate secretion. Nevertheless, intragastric administration of peptide growth factors is limited by acid-proteolytic degradation in the stomach. Large-scale production of human recombinant proteins is still an expensive process. Some of these problems may be overcome by gene transfer of the cDNA of angiogenic growth factors into the lesion directly.

Gene therapy, i.e., direct injection of naked DNA (ND) and using viral vectors for gene transfer with growth factors (e.g., VEGF) has been investigated in the treatment of ischemic diseases in both animal models and clinical settings such as limb ischemia (Tsurumi et al., 1996; Isner et al., 1996) and cardiovascular diseases (Losordo et al., 1998).

The main interest of our research work has been related to the study of vascular factors in mucosal injury and repair (Szabo et al., 1998; Pihan et al., 1988; Szabo, 1989; Morales et al., 1992; Szabo et al., 1997), especially since the ensuing healing of chronic ulcers depends upon the formation of vascularized granulation tissue. Since our pharmacologic experiments demonstrated a potent ulcer healing effect of bFGF, VEGF and PDGF, we now wanted to examine the effects of gene therapy with adenoviral vector (AV) and ND of VEGF and PDGF on the healing of experimental chronic duodenal ulcers.

Methods

Plasmids and adenoviral vector. We purified the naked $VEGF_{165}$ and PDGF plasmids (from Dr. Kevin P. Claffey, Beth Israel Deaconess Medical Center, Boston, MA) transformed to *Escherichia coli* using DNA purification kit (Promega, Madison, WI). Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA) provided AV of human $VEGF_{165,}$ PDGF-B, Lac Z (control) and green fluorescent protein (GFP). The doses of the gene vectors administered in the present study were based on the ischemic and wound healing studies done by Dr. Herlyn's lab (Liechty et al., 1999).

Experimental animals and duodenal ulcer induction. Groups (n = 8-12) of Sprague-Dawley female rats (180-210 g) had unlimited access to food and water. Rats were given cysteamine-HCl (Aldrich, Milwaukee, WI) (25 mg/100 g by gavage x3 with 4 hr intervals) to cause duodenal ulcers on the 1st day. All experiments were carried out in compliance with our Institutional Regulations for Animal Use and Care.

Administration of gene vectors. On the 3rd day after cysteamine administration, we performed a laparotomy while rats were under anesthesia with inhalation of *isoflurane* to evaluate ulcer formation (superficial or perforated ulcers) and randomly divided rats with equally severe duodenal ulcers into control and treatment groups. The controls received during laparotomy intraduodenal (i.d.) instillation of 0.1 ml/rat of Tris-EDTA buffer or 5×10^8 pfu/rat of LacZ AV. Eight treatment groups received 100 μg/rat of ND of VEGF or PDGF x1, i.d. on the 3rd day, 200 μg/rat x2, intravenously (i.v.) on the 3rd and 5th day, or 5×10^8 pfu/rat of VEGF or PDGF AV x1, i.d. or i.v. on the 3rd day (Table 1).

Gross and histologic evaluation of duodenal ulcer healing. *The rats were euthanized by CO_2 inhalation and cervical dislocation on the 7^{th} day (with superficial nonperforated ulcers) and the 14^{th} day (with perforated or penetrated ulcers) after the administration of cysteamine.* The duodenal ulcer crater dimensions were measured in millimeters and the ulcer areas were calculated using the ellipsoid formula. We sampled and fixed the opened stomachs with 2.5 cm duodenum in 10% formalin. Thin sections were stained with hematoxylin and eosin, PAS

staining (to assess the regeneration of mucosa, especially mucus-secreting epithelial cells) or the trichrome techniques (to evaluate the composition of granulation tissue, and smooth muscle). In addition, we harvested and frozen mucosal scrapings of 2.5 cm of proximal duodenum, glandular stomach, jejunum, liver, and kidney from some animals for Western blot analysis, immunohistochemistry and ELISA.

AV-β-galactosidase staining. To examine the localization of the transgenes, β-galactosidase staining was performed at 24 and 48 hr after the i.d. or i.v. administration of AV in the rats with cysteamine-induced duodenal ulcer. *Specimens of the proximal duodenum, stomach, jejunum, liver, spleen, kidney and lung were stained at 37oC with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma, St. Louis, MO) as whole mount tissues or in 10 μm thin sections (Kypson et al., 1998).*

Transfection of recombinant AV carrying the GFP. Subset groups of rats were given a single dose of phosphate-buffered saline (PBS) or 5 x 10^8 pfu/rat of AV-GFP i.d. or i.v. under isoflurane anesthesia on the 3rd day after cysteamine-induced duodenal ulcer and the animals were euthanized 3 days after AV-GFP administration. Specimens of the proximal duodenum, stomach, jejunum, liver, spleen, kidney and lung were harvested and fixed in 4% paraformaldehyde for 24 hr and in 10% sucrose for 24 hr. After tissue sectioning, and hematoxylin and eosin staining, the expressed GFP in the tissues was detected by a fluorescent microscope (AG Heinze, Lake Forest, CA).

Western blot analysis. Proteins **(**100 μg) of mucosal homogenate were separated by 12% SDS-PAGE and then transferred onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The blots were detected using standard Western blot techniques with rabbit polyclonal antibodies against VEGF, PDGF, or bFGF (Santa Cruz Biotechnology, Santa Cruz, CA) followed with using the anti-rabbit IgG horse radish peroxidase conjugate (Santa Cruz Biotechnology). We visualized the immunoreactive proteins using the ECL reagent (Amersham) after exposing the membrane to Hyper film ECL (Amersham).

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Immunohistochemistry. Paraffin tissue sections (10μm) serial to those used for histological analysis were irradiated at 750 W in a microwave oven with 3% hydrogen peroxide in 0.01 M sodium citrate buffer, pH 6.0, dewaxed and immunostained with primary antibodies (Santa Cruz Biotechnology, CA) of anti-VEGF in 1:50 dilution or anti-PDGF in 1:200 dilution, and followed by biotinylated secondary antibodies (Santa Cruz) and a peroxidase-labelled streptavidin-biotin staining technique. To ensure specificity of the antibody, immunoabsorption of the antibodies was performed to provide control.

ELISA. We used human VEGF, PDGF and bFGF immunoassay kits (R&D Systems, Minneapolis, MN) for the measurement of the growth factors in the duodenal mucosa after gene therapy according to the manufacturer's directions. *The human VEGF, PDGF and bFGF proteins have 90% similarity to rat VEGF, PDGF and bFGF (www.ncbi.nlm.nih.gov/blast), so the kits for human growth factors have similar affinity for the rat peptides.* We calculated the concentrations by a ratio (pg/mg) of endogenous VEGF, PDGF or bFGF vs. total proteins.

Data analysis. The statistical significance of differences among group means was calculated using the non-parametric Mann-Whitney U-test. We chose $P<0.05$ or smaller values for statistical significance.

Results

Size of the duodenal ulcers after VEGF or PDGF transgenes. Pooled results from several experiments demonstrate that the ulcer area measured in the controls was around 8 mm^2 (Fig. 1). Therapy with VEGF or PDGF AV caused more prominent healing (Fig. 1A and B) than with ND (Fig. 1C and D) in all treatment groups. The superficial ulcers were significantly decreased ($P<0.05$) by an equivalent dose of VEGF or PDGF ND (Fig.1C), whereas significant healing of severe, perforated ulcers was noted only after VEGF ND treatment (Fig. 1D). In additional experiments, injections of AV of VEGF or PDGF into the duodenal ulcer margins or lumen of duodenum during laparotomy were equally effective in reducing ulcer size.

Macroscopic and microscopic analysis of duodenal ulcers after gene therapy. Representative samples from 4 rats showed the healing of penetrating duodenal ulcers (Fig. 2A). The duodenal ulcer size in the control rats that received the Lac Z AV was markedly larger than in rats that received either VEGF or PDGF AV *by i.d. or i.v. administration*. The ulcers were barely visible or absent after the VEGF or PDGF adenoviral therapy. The histologic appearance of the duodenal ulcers is shown in Fig 2B: the normal duodenal mucosa (hematoxylin and eosin staining) of a control rat is interrupted with sharply demarcated ulcer crater that consists of necrotic and inflamed tissue with some amount of granulation tissue (Fig 2B, a). The PAS staining of control rat ulcer showed no mucus-secreting epithelium over the necrotic ulcer crater (Fig 2B, b). A high power view (x100) of a control ulcer stained with the trichrome technique (which stains differentially epithelium, connective tissue and smooth muscle) demonstrates that the granulation tissue of the ulcer crater had penetrated into the liver. Most of the granulation tissue in untreated ulcer consists of loose granulation tissue (Fig. 2B, c).

Figure 2B (d) is a trichrome staining of a completely healed duodenal ulcer after VEGF AV treatment. The granulation tissue has been reepithelized and the dark blue area indicates a very dense connective tissue replacing the ulcer crater. A few food particles are seen in the granulation tissue, indicating that healing occurred so fast that food was trapped within the healed area. The PAS staining shows that the reepithelized cells also contain mucus-secreting cells, e.g., goblet cells indicating reconstruction of the specialized duodenal epithelium (Fig. 2B, e). A similar morphology indicates that good reepithelization of the ulcer also occurs after PDGF AV treatment (Fig. 2B, f).

Organ transduction of adenoviral vectors. The duodenum after either i.d. or i.v. administration of Lac Z AV was stained blue and the β-galactosidase staining was concentrated in most of the rats around duodenal ulcer margins (Fig. 3). The stomach and jejunum were also stained in both i.d. and i.v. groups but the staining after i.v. injection of AV was stronger than that after i.d. administration. The kidney and, slightly, the spleen were only stained after the i.v. injection, while the liver and the lung lacked staining after either i.d. or i.v. injection of the AV. These findings were confirmed in histologic sections using X-gal staining and by using the AV-GFP reporter gene.

Growth factors within the ulcer area after gene therapy. *After both 7 and 14 day of AV treatment, Western blots of duodenal mucosa showed that most rats treated with either AV VEGF or AV PDGF had increased expression of the predicted 23 kDa VEGF and 30 kDa PDGF although the level of VEGF at 14 days was lower than 7 days (Fig. 4A). Surprisingly, increased expression of endogenous PDGF was also detected in the samples after gene therapy of AV VEGF, and increased VEGF was seen in samples following gene therapy with AV PDGF as well (Fig. 4A). In 14 days after ND, VEGF expression was less than 7 days after either ND VEGF or ND PDGF treatment (Fig 4B). No elevation of PDGF and VEGF was seen in samples from stomach, jejunum, liver, spleen, kidney and lung (Fig. 4C).*

Light microscopic examination of immunohistochemistry staining of duodenal sections demonstrated an increased expression of VEGF or PDGF in the granulation tissue of the ulcer crater, especially in endothelial cells and macrophages. Furthermore, VEGF and PDGF positivity was also seen in monocytes in the duodenal lamina propria of rats after gene therapy with VEGF or PDGF, whereas, this was not seen in the controls (Fig. 5)

The concentration of endogenous VEGF and PDGF was increased by 40-50% in the duodenal mucosa after 7 and 14 days with ND or AV of VEGF or PDGF as measured by ELISA (Fig. 6). This confirms the findings by the Western blots and IHC. Surprisingly, the levels of endogenous bFGF were also increased by 50-70 % mainly after ND or AV of PDGF treatment in 7 days.

Discussion

The present study showed that gene therapy with ND or AV of PDGF and VEGF after local (intraduodenal instillation) or systemic (intravenous injection) administration accelerated the healing of experimental duodenal ulcers. The AV seemed to be more effective, especially with VEGF than the ND, *although these results are not directly comparable since the delivery vehicle (AV vs. ND) of gene therapy was different.* The dose of 5×10^8 pfu in the present study did not induce broad transduction in distal organs. Furthermore, after the local administration the AV transduction was only localized in the duodenum, stomach and jejunum. In addition, our results showed that a 10-fold less than the regular dose (5×10^8 pfu) was also effective in accelerating duodenal ulcer healing (Szabo et al., 2001). Thus, the possible risks induced by adenovirus would be decreased by using a lower dose and local administration in gene therapy for duodenal ulcer.

Although the ND, unlike the AV, does not result in a strong transfection in cells (e.g., it is taken up chiefly by free diffusion), numerous studies have successfully demonstrated transduction of genes into target cells with a variety of vectors, and have provided 'proof-in-principle' that gene transfer can result in prolonged *in vivo* expression of transduced genes and an excess of cellular accumulation located in the targeting tissues (Davidson et al., 2001; Prince, 1998). Intramuscular injection of ND encoding VEGF augmented development of collateral vessels and tissue perfusion in limb ischemia (Tsurumi et al., 1996). Local administration of ND encoding VEGF markedly improved the healing of ischemic diseases such as myocardial infarcts (Losordo et al., 1998; Giordano et al., 1996) and peripheral ischemic ulcers (Isner et al., 1996; Takeshita et al, 1996). These findings may be cautiously interpreted to indicate that intramuscular injection of ND achieves constitutive overexpression of VEGF sufficient to induce therapeutic angiogenesis in patients with critical limb ischemia.

Adenovirus has been widely used as a vector for gene transfer because it possesses relatively high transduction efficiency into a variety of cells in the postmitotic stage. This may be the main reason why duodenal ulcer healing after gene therapy with adenoviral vector of VEGF or PDGF was better than the ND gene therapy in the present study. The major limitations of gene therapy with viral vectors, however, are the lack of sustained expression, because the viral DNA does not integrate into the host genome. Antigenicity against viral proteins by both humoral and cytotoxic T-lymphocytes and possible toxicity at high dose could be other limiting factors (Prince, 1998).

Of particular interest in the present study is that the local or systemic administration of either VEGF or PDGF gene therapy increased the levels of both VEGF and PDGF in the duodenal mucosa as demonstrated by both Western blotting analysis and immunohistochemistry staining. *VEGF and PDGF levels were increased early, e.g., more after 7 days than 14 days, probably representing a rapid response to tissue injury. Furthermore, ND PDGF increased the local expression of PDGF and decreased ulcer size only in 7 days but not after 14 days.* The exact mechanisms are not clear. Some investigations have showed that PDGF may induce VEGF production in smooth muscle cells (Iruela-Arispe et al., 1995; Brogi et al., 1994; Wang et al., 1999; Kronemann et al., 1999). PDGF is thought to induce VEGF through activation of PDGF ß receptor expressed on endothelial cells (Wang et al., 1999). The expression of PDGF ß receptor on endothelial cells is a prerequisite for PDGF to elicit a direct effect on neovascularization. Several research groups have reported up-regulation of the PDGF ß receptors for the capillary endothelial cells in human carcinoid tumors, in wounds, and inflammatory tissues (Reuterdahl, 1991; Reuterdahl, 1993; Funa et al., 1990). These results suggest that PDGF may have a direct effect on endothelial cells undergoing angiogenesis. In this regard, PDGF may act as a mitogen and directly stimulate endothelial cell proliferation (Battegay et al., 1994), or it may induce VEGF expression in endothelial cells, which in turn causes an autocrine stimulation through VEGF receptors (Ferrara and Davis-Smyth, 1997).

The histologic and histochemical examination of ulcer healing in the present study has interesting implications. Namely, many mucus-secreting cells such as goblet cells appeared in the early stages of reepithelization of the mucosa after gene therapy (e.g., some food particles were trapped in the granulation tissues under the new epithelium), while the new flat epithelium on the edge of the control rat ulcer did not contain these mucus-secreting cells. We did not see this extensive and rapid regeneration of specialized epithelial cells in our previous studies using not only antisecretory drugs but not even after peptide treatment with growth factors such as VEGF, PDGF or bFGF.

We also detected in the present study elevated bFGF concentration mainly after treatment with naked DNA or adenoviral vector of PDGF. It is unclear how PDGF modulates bFGF induction but a recent study demonstrated that bFGF could affect PDGF production by specific upregulation of the PDGF α receptor in vascular smooth muscle cells (Russo et al., 2002). Our previous studies demonstrated that oral treatment with either naturally occurring bFGF or its acid

resistant mutein (bFGF-CS223) at 100 ng/100 g twice daily for 3 weeks accelerated the healing of cysteamine-induced chronic duodenal ulcer (Szabo et al., 1991; Szabo et al., 1994). Endogenous bFGF was visualized by immunostaining techniques in the rat duodenum after cysteamine administration, and bFGF immunoreactivity was time-dependently reduced in the mucosa, submucosa and muscularis propria (Szabo et al., 1993; Kusstatscher e al., 1993; Sakoulas et al., 1993).

In summary, gene therapy with ND or AV of VEGF or PDGF significantly accelerated the healing of superficial, penetrated or perforated chronic duodenal ulcers 7 and 14 days after the administration of duodenal ulcerogen cysteamine. We found the fast reepithelization and mucosal regeneration after AV of VEGF or PDGF gene therapy included new specialized, e.g., mucus-secreting PAS-positive cells. We detected increased levels of endogenous VEGF and PDGF by Western blotting, ELISA and immunostaining in duodenal tissues after either VEGF or PDGF gene therapy. Broad expression of the adenoviral transgenes in distal organs was seen only after i.v. administration of AV. Thus, VEGF and PDGF gene therapy, especially with adenoviral vector, seems to be a new modality of achieving a rapid duodenal ulcer healing.

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Footnotes

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Legends for figures:

Figure 1. The size of duodenal ulcers in 7 and 14 days after AV or ND of VEGF or PDGF compared with controls. * $P < 0.05$ and ** $P < 0.01$. n=6-12.

Figure 2. A: The gross appearance of cysteamine-induced duodenal ulcers (arrows) after i.d. or i.v. administration of AV of Lac Z (control), VEGF or PDGF in 7 or 14 days: the ulcers are reduced or not visible after gene therapy. B: (a) Duodenal ulcer in a control rat (hematoxylin and eosin): the normal duodenal mucosa (i) is interrupted with sharply demarcated ulcer crater which consists of necrotic (ii) and granulation tissue (iii). (b) A control duodenal ulcer stained with the PAS method which delineates mucus secreting goblet cells (i) and Brunner glands (ii) in the submucosa. The new flat epithelium on the edge of control ulcer does not contain mucus secreting cells (iii). (c) Another control ulcer stained with the trichrome technique: the epithelium (i) and liver (ii) (penetrating ulcer into the liver) are red. The granulation tissue with dense collagen is blue (iii), and the loose granulation tissue is greenish-blue (iv). (d) Trichrome staining of a healed duodenal ulcer after AV of VEGF: Reepithelization (i) of granulation tissue which contains dense collagen (ii) and multinucleated giant cells (iii). (e) PAS staining of a completely healed duodenal ulcer 14 days after AV PDGF treatment: Goblet cell-containing reepithelization (i) over the *food particles (ii) and multinucleated giant cells (iii) trapped by* dense granulation tissue. (f) Similar, healed duodenal ulcer stained with PAS: new epithelium with mucus secreting goblet cells (i), dense granulation tissue with food particles (ii) and multinucleated giant cells (iii).

Figure 3. Localization (blue staining) of transducted *Lec Z AV* in multiple organs 48 hr after i.v. or i.d. administration *with a single dose of* $5x10^8$*pfu/ra.* Du: duodenum, St: stomach, Je: jejunum, Li: liver, Sp: spleen, Ki: kidney, and Lu: lung.

Figure 4. A: VEGF and PDGF measured by Western blot in the duodenal mucosa 7 and 14 days after AV or ND of VEGF or PDGF treatment. B: VEGF and PDGF measured by Western blot in the other organs 7 days after AV of Lec Z, VEGF or PDGF treatment. St: stomach, Je: jejunum, Li: liver, Sp: spleen, Ki: kidney, and Lu: lung.

Figure 5. Expression of VEGF and PDGF detected by IHC in the duodenum 7 or 14 days after induction of duodenal ulcer by cysteamine and treatment with ND or AV of VEGF or PDGF. Growth factors were not detected (no brown staining) in duodenal ulcers of control rats after either anti-VEGF (a) or anti-PDGF (b) antibodies. In ulcer samples after VEGF or PDGF gene therapy, positive staining of endothelial cells, macrophages and monocytes was seen (arrows): c= VEGF staining after ND-PDGF 100ug, i.d.; d = VEGF staining after ND-VEGF 200ug i.v.; e = PDGF staining after ND-PDGF 200ug i.v.; f = PDGF staining after ND-PDGF 100ug i.d.; g = VEGF staining after AV-VEGF 5 x 10^8pfu i.d.; h = PDGF staining after AV-PDGF 5 x 10^8pfu i.d.

Figure 6. Concentration of VEGF, PDGF and bFGF in duodenal mucosa measured by ELISA 7 days after induction of duodenal ulcer induced by cysteamine and treatment with naked DNA (ND) or adenoviral vector (AV) of VEGF or PDGF.

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Table 1. Treatment groups of rats with cysteamine-induced duodenal ulcers

Group	Vector	Dose	Volume (ml)	Route	Frequency	Time of injection
1	AV-Lec Z	5 x 10^8 pfu/rat	0.1	i.d.	x1	3rd day
2	AV-VEGF	5 x 10^8 pfu/rat	0.1	i.d.	x1	3rd day
3	AV-VEGF	5 x 10^8 pfu/rat	0.1	i.v.	x1	3rd day
4	AV-PDGF	5 x 10^8 pfu/rat	0.1	i.d.	x1	3rd day
5	AV-PDGF	5 x 10^8 pfu/rat	0.1	i.v.	x1	3rd day
6	TE buffer		0.1	i.d.	x1	3rd day
7	ND-VEGF	100 μg/rat	0.1	i.d.	x1	3rd day
8	ND-VEGF	200 μg/rat	0.1	i.v.	x2	3rd, 5th day
9	ND-PDGF	100 μg/rat	0.1	i.d.	x1	3rd day
10	ND-PDGF	200 μg/rat	0.1	i.v.	x2	3rd, 5th day

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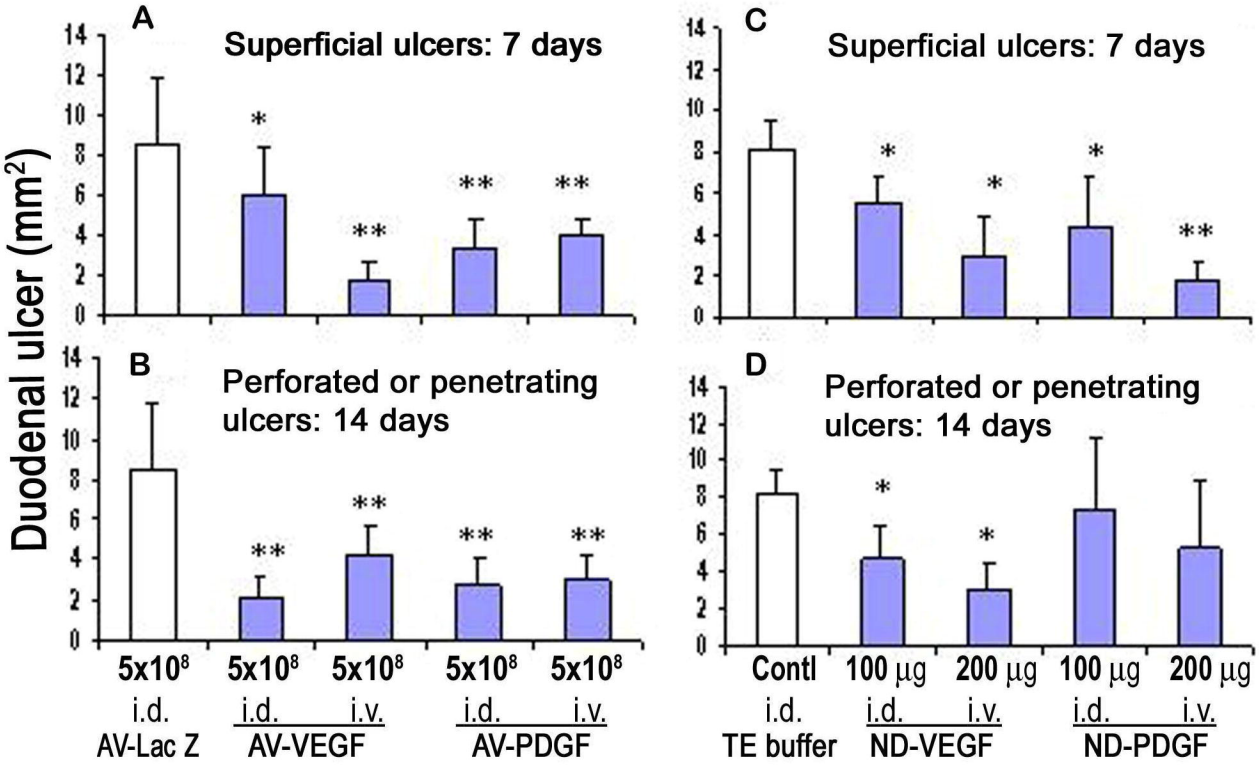

Figure 1

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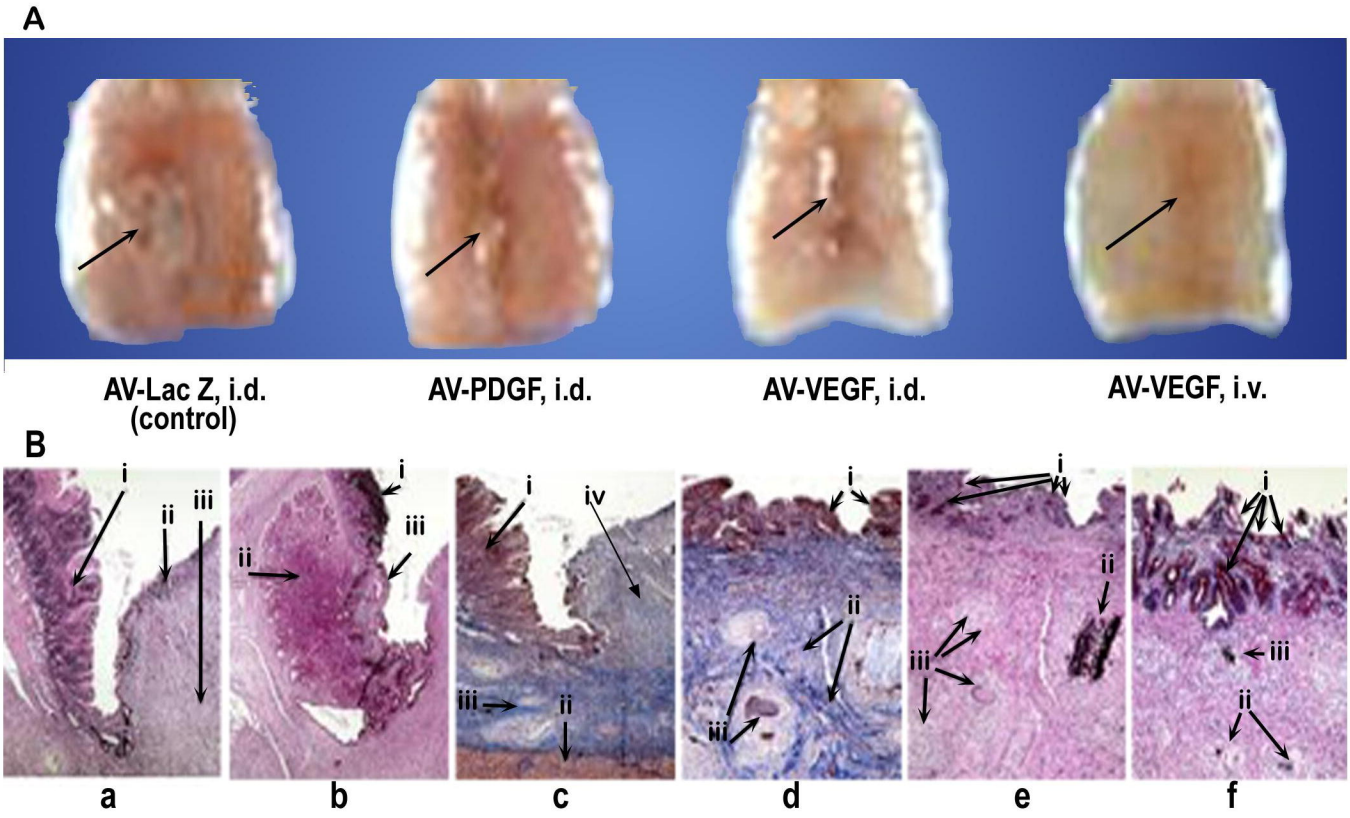

Figure 2

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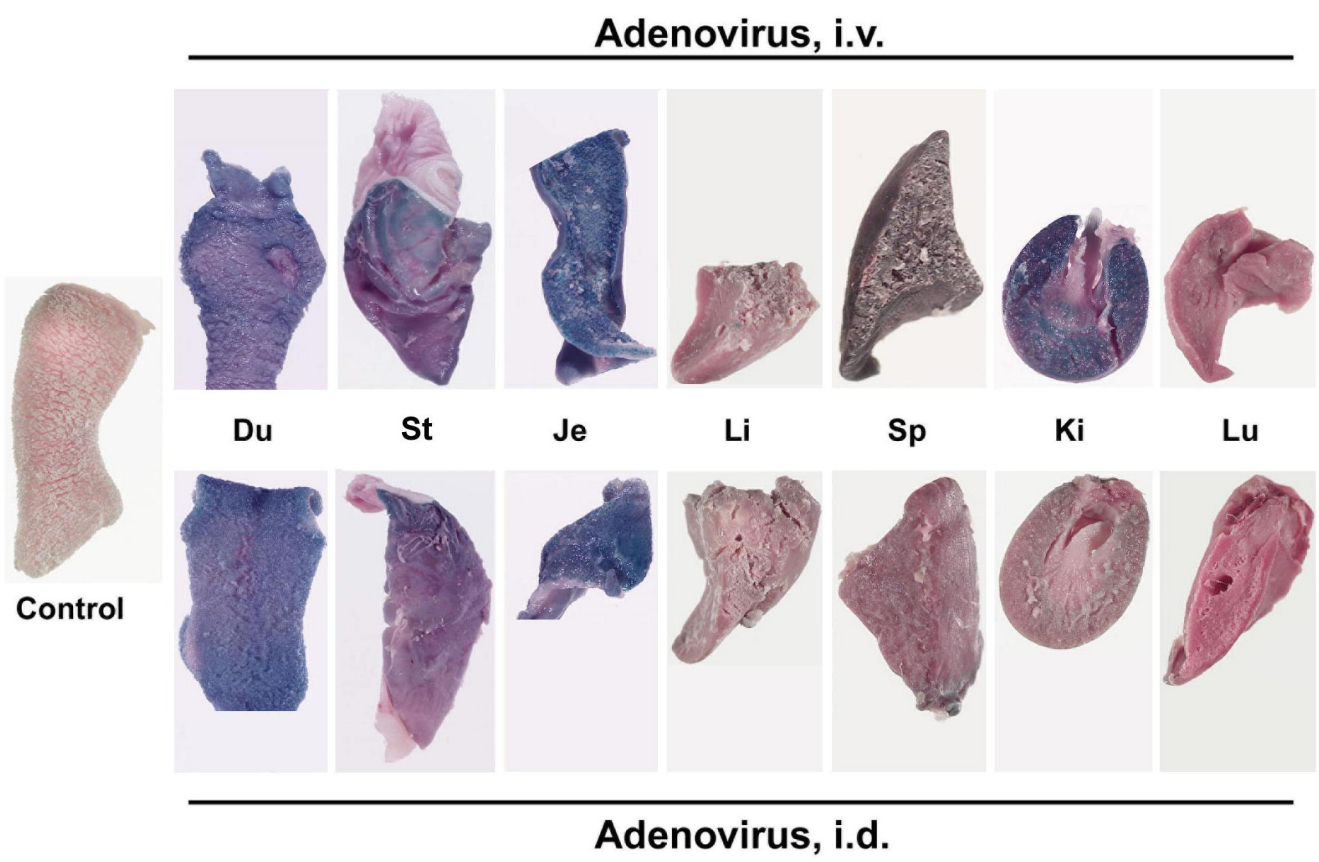
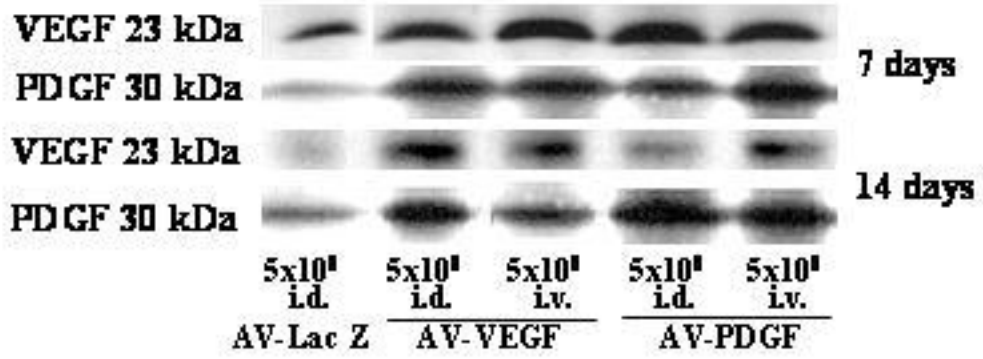
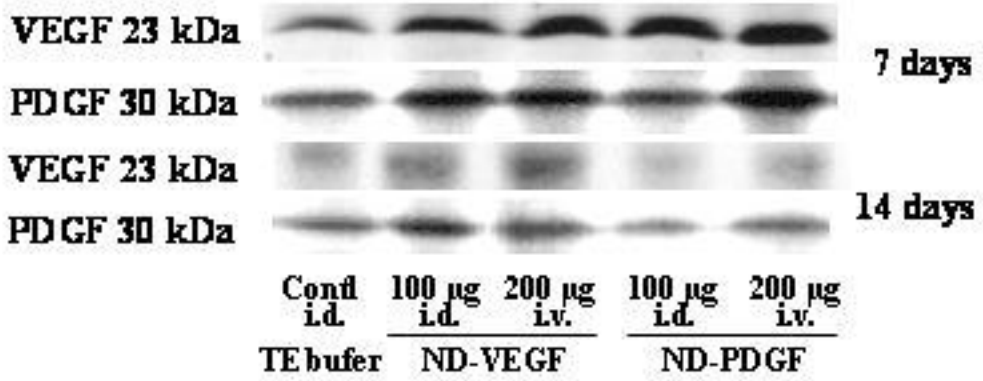


Figure 3

A


VEGF 23 kDa
PDGF 30 kDa
VEGF 23 kDa
PDGF 30 kDa
7 days
14 days
5x10⁸ i.d.
5x10⁸ i.d.
5x10⁸ i.v.
5x10⁸ i.d.
5x10⁸ i.v.
AV-Lac Z
AV-VEGF
AV-PDGF

B


VEGF 23 kDa
PDGF 30 kDa
VEGF 23 kDa
PDGF 30 kDa
7 days
14 days
Contl i.d.
100 µg i.d.
200 µg i.v.
100 µg i.d.
200 µg i.v.
TE bufer
ND-VEGF
ND-PDGF

C

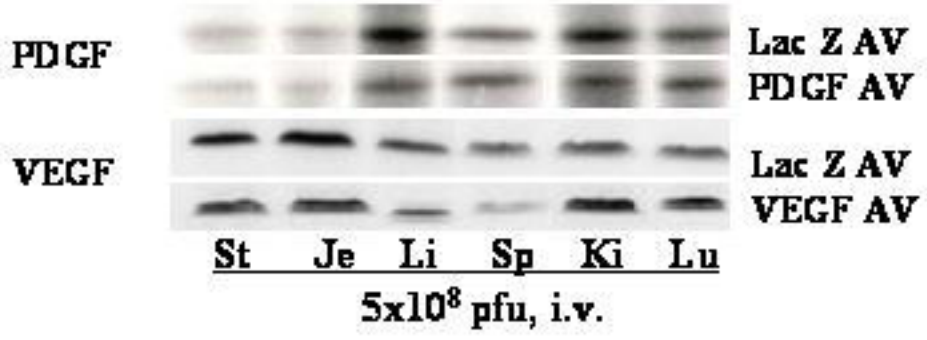

PDGF
VEGF
Lac Z AV
PDGF AV
Lac Z AV
VEGF AV
St
Je
Li
Sp
Ki
Lu
5x10⁸ pfu, i.v.

Figure 4

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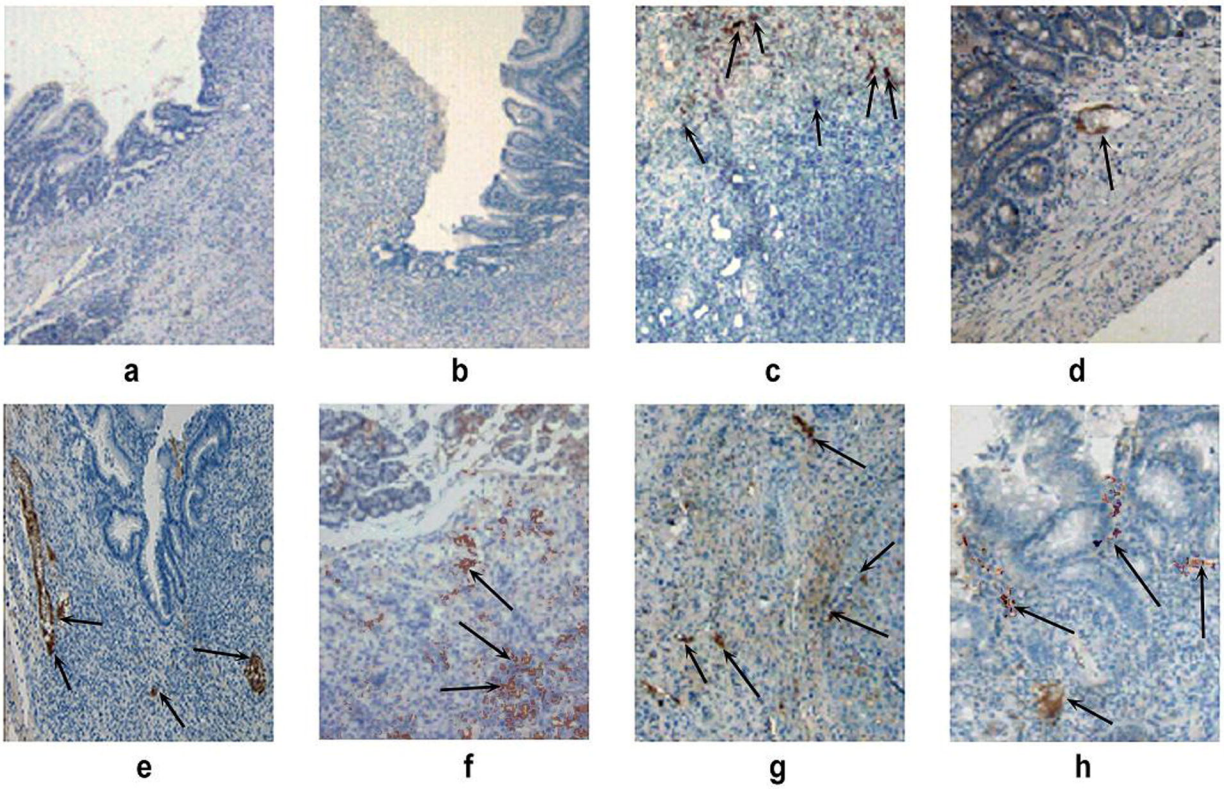

Figure 5

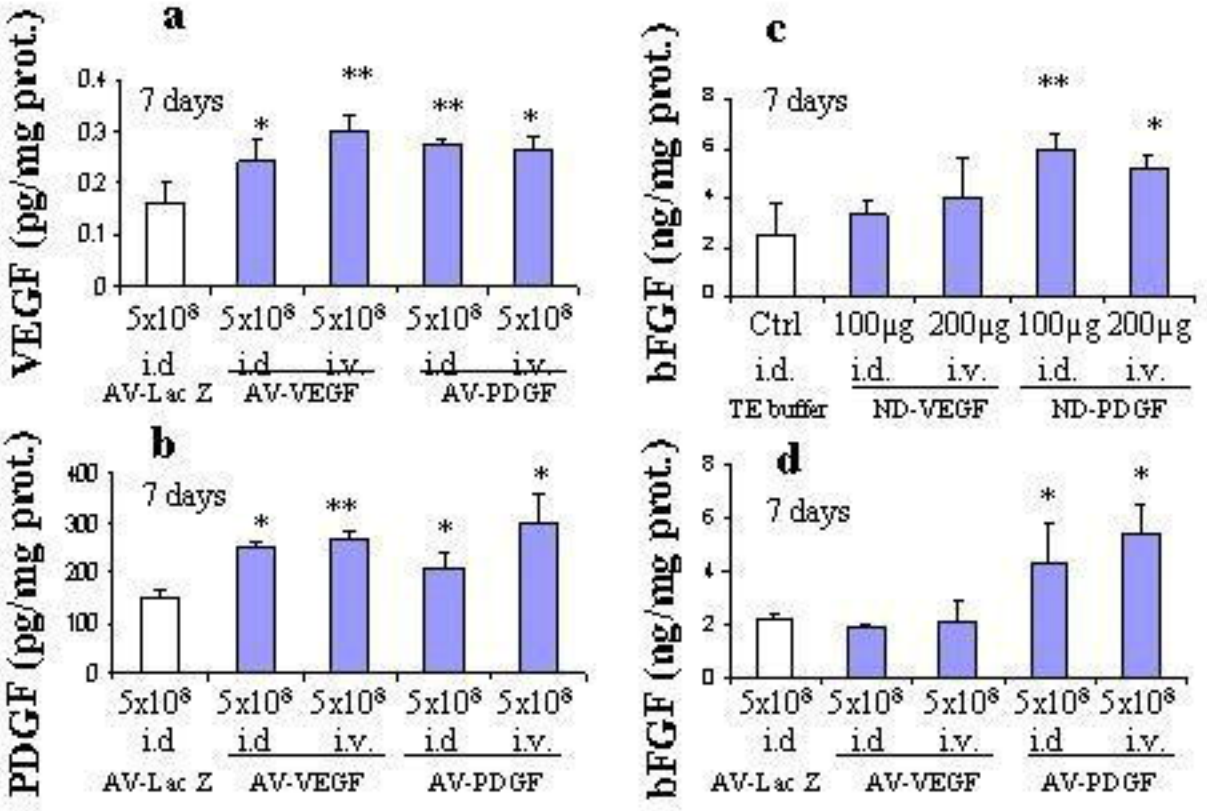
a
VEGF (pg/mg prot.)
7 days
0.4
0.3
0.2
0.1
0
*
**
**
*
5x10⁸ 5x10⁸ 5x10⁸ 5x10⁸ 5x10⁸
i.d i.d i.v. i.d i.v.
AV-Lac Z AV-VEGF AV-PDGF
b
PDGF (pg/mg prot.)
7 days
400
300
200
100
0
*
**
*
*
5x10⁸ 5x10⁸ 5x10⁸ 5x10⁸ 5x10⁸
i.d i.d i.v. i.d i.v.
AV-Lac Z AV-VEGF AV-PDGF
c
bFGF (ng/mg prot.)
7 days
8
6
4
2
0
**
*
Ctrl 100µg 200µg 100µg 200µg
i.d. i.d. i.v. i.d. i.v.
TE buffer ND-VEGF ND-PDGF
d
bFGF (ng/mg prot.)
7 days
8
6
4
2
0
*
*
5x10⁸ 5x10⁸ 5x10⁸ 5x10⁸ 5x10⁸
i.d i.d i.v. i.d i.v.
AV-Lac Z AV-VEGF AV-PDGF

Figure 6