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Review

Antiangiogenic gene therapy in inhibition of metastasis★✪

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This short review attempts to demonstrate the usefulness of antiangiogenic gene therapy in achieving inhibition of growth in experimentally-induced metastases. Certain normal tissues (for example skeletal muscle) may be used *in vivo*, after genetic modification, as a "bioreactor", able to produce and secrete into the bloodstream proteins known to exert antiangiogenic effects. By inhibiting neoangiogenesis these proteins would thus prevent the development of metastases. The review discusses also the perspectives of antimetastatic therapy based on certain types of allogenic cells (for example myoblasts and fibroblasts) that had been genetically modified and then microencapsulated. The strategy of encapsulation is aimed at protecting the modified cells secreting antiangiogenic factors from being eliminated by the immune system.

Secretion of antiangiogenic proteins by these microencapsulated cells can be controlled with inducible promoters. Antiangiogenic genes remaining under the transcriptional control of such promoters may be switched on and off using antibiotics, such as tetracycline derivatives, or steroid hormones.

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Abbreviations: ECM, extracellular matrix; MMP, matrix metalloprotease(s); PBS, phosphate-buffered saline.

THE PROCESS OF METASTASIS FORMATION. BASIC STEPS IN THE DEVELOPMENT OF METASTASES

The appearance of metastases in latter stages of neoplastic disease results in unfavorable clinical prognostics; metastatic cells become resistant to the majority of known drugs and treatment strategies (Liotta *et al.*, 1993; Engers & Gabbert, 2000; Condeelis *et al.*, 2000; Chambers *et al.*, 2000; Bani & Giavazzi, 2000).

Formation of metastases is a multi-step event that may be arbitrarily divided into four stages, each comprising several sub-stages. During the first stage neoplastic cells capable of migration appear at the primary site (primary colony) (Liotta *et al.*, 1993). These cells exhibit altered cell–to–cell interactions (they lack homotype relations *via* E-cadherins) as well as altered interactions with the extracellular matrix (ECM) (Engers & Gabbert, 2000). An important role in these altered relations between neoplastic and normal cells as well as the extracellular matrix is ascribed to adhesion molecules such as integrins, immunoglobulin (Ig) superfamily, P-, E- and L-selectins, ICAM-1, VCAM-1, NCAM, DCC, Lewis family blood group antigens, mucins, glycosphingolipids, CD 44 molecules, Lu-ECAM-1 and others (Bani & Giavazzi, 2000). The migrating cells are also able to secrete and/or activate proteolytic enzymes involved in cell locomotion across the ECM (matrix metalloproteases, MMP) (Engers & Gabbert, 2000).

During the second stage of metastatic spread (intravasation), migrating metastatic cells penetrate into the lumen of blood vessels or, rarely, lymphatic vessels. The cells are passively carried with the bloodstream and become arrested in microvessels of diameter smaller than their own (Chambers *et al.*, 2000). The arrested cells would frequently become deformed. They might attach to blood vessel walls, most likely by adhesive interactions. Alternatively, single metastatic cells could be arrested in vessels with a diameter larger than their own (Al-Mehdi *et al.*, 2000). In that case interactions of adhesive nature would definitely be responsible for the cells sticking onto the blood vessel walls.

Extravasation is the third step of metastasis during which cancer cells leave the blood or lymph vessels. Their penetration through the vessel wall does not cause mechanical damage to the latter. Twenty four hours from the arrest of metastatic cells in microcirculation 80% of them undergo extravasation (Chambers *et al.*, 2000). Data exist indicating that metastatic cells adhering to the walls of pulmonary vessels do not actually extravasate but begin to proliferate and form microcolonies entirely within the blood vessels. It is believed that in such case the vessel wall is destroyed after some time resulting in the colony growth outside of it (metastasis) (Al-Mehdi *et al.*, 2000).

The fourth (last) stage of the metastatic process involves formation of secondary colonies called metastases. Extravasating cancer cells are thought to migrate towards arterioles (Chambers *et al.*, 2000). Direct physical contact is postulated between neoplastic cells and arterioles. Of decisive importance at this stage of metastasis may be chemotactic interactions; owing to the presence of specific receptors cancer cells are believed to migrate towards the source of specific chemokines. These effector molecules are believed to be responsible for the observed preferences in metastatic spread to specific organs; for example melanoma metastasizes foremost into lungs while breast cancer into bones, etc. (Müller *et al.*, 2001).

From among many extravasating cells only a few are able to produce a micrometastatic colony (Chambers *et al.*, 2000). The majority of solitary cancer cells, as well as metastases, do not survive as the lack of certain growth factors in the newly reached environment may induce apoptosis in these cells (Wong *et al.*, 2001). In all likelihood apoptosis of metastatic cells is responsible for the phenomenon called

metastatic inefficiency. However, a considerable number of solitary extravasated cells and micrometastases remain dormant. Under this condition there exists a unique equilibrium in metastasizing cells between apoptosis and proliferation (Holmgren *et al.*, 1995). Only a tiny fraction of such dormant cells or micrometastases may further evolve into macrometastases. Chambers *et al.* (2000) have calculated that only one in a hundred micrometastases survives and forms metastasis. An exceptional and probably decisive role in the initiation and maintenance of metastases is played by angiogenesis. Activation of proangiogenic factors by metastatic cells results in the development of microvasculature that promotes secondary tumor growth.

Table 1 summarizes the discussed stages of metastasis formation and lists the better known molecular events that can become a target of antimetastatic therapy.

ANTIANGIOGENIC STRATEGY IN ACHIEVING INHIBITION OF METASTASIS

Several data indicate that molecular therapeutic interventions targeted at metastatic spread of cancer cells are feasible above all:

- at the stage involving the appearance of mobile cells (i.e. cells migrating in the ECM) (Dimitroff *et al.*, 1998);
- at the stage involving growth initiation of dormant cells or micrometastases (McDonnell *et al.*, 2000).

Suppression of neoplastic cells' mobility could be brought about, for example, by inhibiting metalloprotease (MMP) activity, increasing the activity of MMP protein inhibitors, blocking activation of $\alpha_v\beta_3$ integrins, blocking signal transduction by MAP kinases or decreasing RAGE – amphoterin interactions that accompany pseudopodia formation.

However, the antimetastatic therapeutic interventions likely to be biologically appropriate and clinically accessible rather involve strategies suppressing growth initiation of dormant micrometastases (Folkman, 1996) (see also Table 1). It appears that angiogenesis inhibitors such as endostatin or angiostatin are best suited to such purpose since they inhibit angiogenesis within primary tumors as well as micrometastases (Folkman, 1998; Sim *et al.*, 2000).

The antimetastatic therapy must meet the following conditions:

- the therapy ought to be systemic in nature, not merely local (i.e. it should not be confined to a single organ or site);
- the effect of therapeutic factors (angiogenesis inhibitors) should be long-term (chronic) since primary tumors constantly shed metastasizing cells and dormant cells and micrometastases can become activated at any time during the course of the disease;
- the level of blood-circulating inhibitor should be high enough to prevent proliferation of endothelial cells in peritumoral vessels;
- the long-term presence of protein inhibitors of angiogenesis should not cause deleterious side effects.

Gene therapy pretends to be a superior delivery alternative of angiogenetic inhibitors to a cancer-afflicted organism (Folkman, 1998; Kong & Crystal, 1998; Feldman & Libutti, 2000). Genetic modification of certain normal cells resulting in their ability to produce and secrete angiogenesis inhibitors is a less expensive as well as a simpler delivery method compared to production, purification and administration of recombinant angiostatic proteins.

In order for the antiangiogenic gene therapy to become clinically realistic in the future some additional conditions must be met:

- the number of genetically engineered cells containing the transgene and secreting the angiostatic protein should be as high as possible;
- the transgene should persist in the "producer" cells for a sufficiently long time; se-

Table 1. Stages of metastasis formation and their molecular basis that can be used in therapy

Stage of metastasis formation	Molecular basis	Antimetastatic therapy
STAGE 1:		
–primary tumor neoplastic cells acquire mobility	–loss of cell-to-cell attachments (CAM, cadherins (Bani & Giavazzi, 2000))	–?
	–increased cell migration, overexpression of RhoC fibronectin, thymosine $\beta 4$ (Clark *et al.*, 2000)	–inhibiting RhoC expression (?)
	–increased cell migration, increased MMP activity (Engers & Gabbert, 2000)	–MMP inhibitors
	–pseudopodia formation (RAGE-amphoterin (Taguchi *et al.*, 2000)	–inhibiting transduction of MAPK signals (?) inhibiting RAGE-amphoterin interactions (?) ("signal transduction therapy")
STAGE 2:		
–mobile neoplastic cells penetrate circulation (intravasation)		–
–cells present in bloodstream		
–adhesion (sticking) of cells in microcirculation (arrest in microvessels)	–interaction of neoplastic cells with platelets, participation of $\alpha_v\beta_3$ integrins in adhesion (Felding-Habermann *et al.*, 2001)	–inhibiting "activation" of $\alpha_v\beta_3$ integrins (?)
	formation of tumor-platelet-leukocyte emboli participation of selectins in adhesion (Kim *et al.*, 1998)	–?
	–specific chemokines, overexpression of chemokine receptors in neoplastic cells (Müller *et al.*, 2001)	–chemoprevention (chemokine antagonists?)
–deformation of cells	–?	–
STAGE 3:		
–extravasation (penetration into surrounding tissues)	–?	
–neoplastic cells outside blood vessels	–?	
–migration towards the closest arteriole	–?	
–adhesion to arteriole	–?	
STAGE 4:		
–proliferation of neoplastic cells, metastasis formation		–inhibition of angiogenesis

cretion of the angiostatic protein should be long-lasting.

GENETICALLY MODIFIED NORMAL CELLS AS A "BIOREACTOR" PRODUCING ANTIANGIOGENIC FACTORS

Genetic modification of normal cells (muscle and liver cells, fibroblasts, etc.) that subsequently serve as a specific "bioreactor" producing and secreting antiangiogenic factors can take place either *in vivo* or *ex vivo*.

The *in vivo* approach most frequently makes use of adenoviral vectors that show a specific tropism for hepatic cells (Chen *et al.*, 2000). In animal models, *in vivo* transfer often involves naked plasmid DNA injected into striated muscle cells (Hartikka *et al.*, 1996). Recently, electrotransfer (electroporation) has been introduced as an efficient method of gene transfer into muscles (Mir, 2000).

Ex vivo transfer also makes use of allogenic fibroblasts (i.e. fibroblasts from the same species individuals). Such fibroblasts, transduced with retroviruses (whereby the transgene is incorporated into the cellular genome), have been subsequently transferred back into experimental animals (Tanaka *et al.*, 2001).

To achieve inhibition of experimental metastases in a mouse model we also have attempted an antiangiogenic strategy in our laboratory. We used the gene encoding endostatin, one of the better known and studied antiangiogenic factors (O'Reilly *et al.*, 1997). We cloned this gene into the pVR1012 vector. Its elements were assembled so as to maximize transgene expression in muscle cells (Hartikka *et al.*, 1996). We investigated the antiangiogenic properties of the protein encoded by the gene we cloned. We showed that this gene, when transferred directly into primary tumors, became expressed and the antiangiogenic protein encoded clearly inhibited growth of such tumors (Szary & Szala, 2001) (Fig. 1). In our murine model of anti-

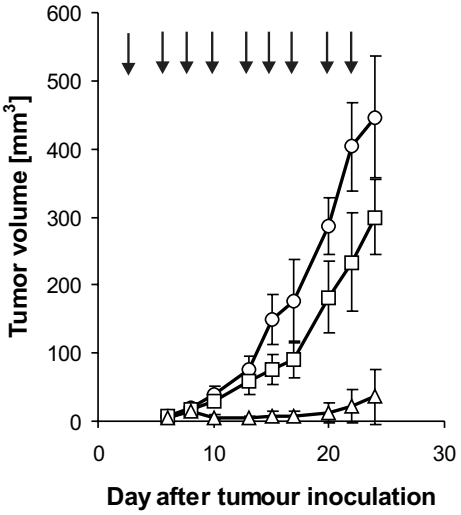

Figure 1. Renca tumor growth inhibition following injection of naked plasmid DNA encoding endostatin (reprinted with permission from: Intra-tumoral administration of naked plasmid DNA encoding mouse endostatin inhibits renal carcinoma growth, Szary J, Szala S, *Int J Cancer*, 2001, Wiley-Lioss, Inc. a subsidiary of John Wiley & Sons, Inc.).

Experimental groups consisted of 5 mice. Ringer's solution (open circles), VR1012 plasmid (open squares) and VR1012/endo plasmid (open triangles) were injected as indicated by arrows. Therapy was started on the third day of the experiment. Values represent mean tumor volume ± S.D. The difference between the endostatin plasmid-treated group and the remaining groups was statistically significant from day 10 to day 24 of the experiment ($P < 0.03$; Mann-Whitney U-test). There was no statistically significant difference between the two control groups. In the endostatin plasmid-treated group, only one mouse developed a tumor; the remaining four mice remained tumor-free for over 100 days. Similar results were obtained in a duplicate experiment.

metastatic therapy, four days following intravenous injection of an appropriate number of melanoma cells (such cells invade lungs and form metastases), plasmid DNA carrying the endostatin-encoding gene was transferred into striated muscle cells by means of electroporation. The conditions of electrotransfer, i.e. impulse duration, voltage, etc., had been previously optimized to maximize the propor-

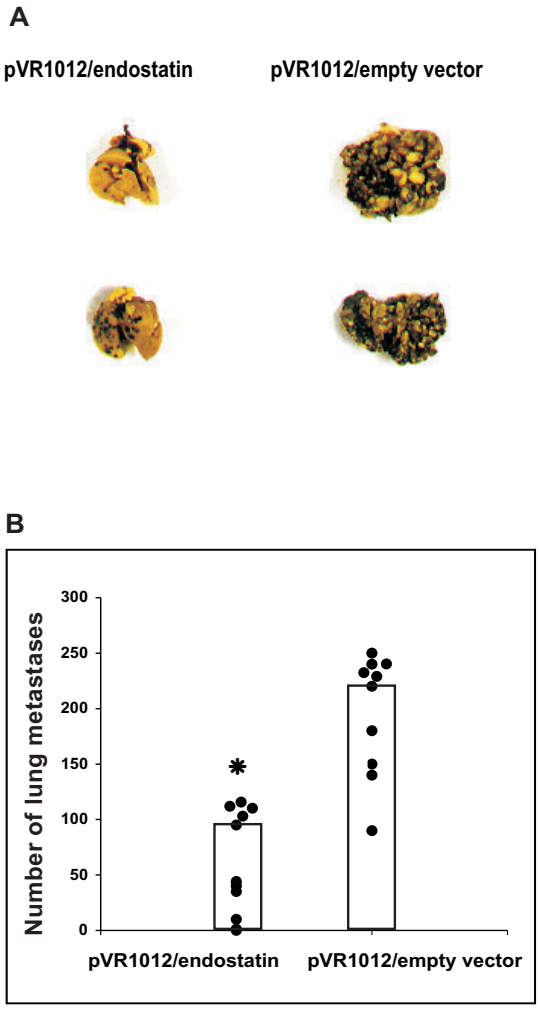

Figure 2. Influence of systemically secreted endostatin upon growth of experimentally-induced metastases of B16(F10) melanoma in lungs of C57BL/6 mice.

C57Bl/6 mice were inoculated *via* the tail vein with a suspension of B16(F10) cells (1×10^6 cells/100 μL PBS^- per animal). On the 4th day after inoculation with the tumor cells, 10 μg of either plasmid DNA containing the endostatin gene (pVR1012/endostatin) in100 μL PBS^- or 10 μg of plasmid DNA without the endostatin gene (pVR1012) in 100 μL PBS^- was injected into both hind limb tibial muscles. Legs were then covered with ultrasonography gel and electroporated. Electroporation conditions: 5 impulses, 900 V, impulse duration 100 μs, distance between caliper electrodes 5 mm. After 7 days the electrotransfer was repeated. The experiment was terminated after 3 weeks from time of inoculation. Mice were sacrificed and lungs fixed in Bouin's solution. **A,** typical view of lungs from mice electrotransferred with either endostatin gene construct (left) or empty construct (right); **B,** number of metastatic foci in lungs of treated and control mice. Significant difference from empty plasmid, $P < 0.0003$; Mann-Whitney U-test.

tion of transfected muscle cells (Cichoń *et al.*, in press).

Figure 2 shows experimental data from our studies. It is clear that the endostatin gene was indeed transferred into muscles by means of electroporation resulting in its expression and that the level of blood-secreted endostatin is sufficient to prevent growth of experimental tumors in mouse lungs. Thus, our model of genetically modified muscle cells with a relatively long persisting transgene (up to several months) is actually a sort of "bioreactor" producing a therapeutic protein (Blezinger *et al.*, 1999).

DEVELOPMENTAL ISSUES

Cells genetically modified *ex vivo* (both allogenic and xenogenic) when microencapsulated, appear particularly well suited to the requirements of antimetastatic therapy. Microencapsulation protects them from being eliminated by the host's immune system (Brauker *et al.*, 1998; Löhr *et al.*, 1998). Such genetically modified and capsule-enclosed cells implanted (most often) intradermally may produce therapeutic proteins for up to several weeks. The use of encapsulated cells (therapeutic protein-secreting fibroblasts, myoblasts etc.) is being tested in the treatment of such genetically-determined diseases as hemophilia B (Hortelano *et al.*, 1999), β-thalassemia (Bachoud-Lévi *et al.*, 2000), and Huntington disease (Dalle *et al.*, 1999). A particularly attractive application strategy may be conceived by combining microencapsulation with regulatable secretion of therapeutic proteins expressed from genes that remain under transcriptional control of promoters induced by such drugs as doxycycline or mifepristone (Serguera *et al.*, 1999).

A sufficiently precise control of gene expression involving antiangiogenic factors (and thereby their secretion by cells) may be achieved when the coding sequences are placed under transcriptional control of the

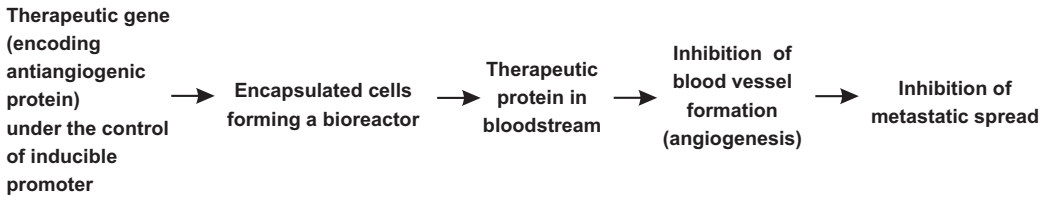

Figure 3. Use of genes encoding antiangiogenic proteins in antimetastatic therapy.

Genetically-modified normal cells as bioreactors producing therapeutic proteins and secreting them into the bloodstream.

so-called inducible promoters (Miller & Whelan, 1997; Maxwell & Cripe, 2000; Fussenegger, 2001). These promoters are activated by transcription factors that in turn require for their activity suitable inducers, for example tetracycline antibiotics or steroid hormones. A non-active gene encoding a therapeutic protein becomes transcriptionally active in the presence of appropriate inducer. Actually, the transcriptional activation systems are binary in nature and comprise two different genes. The first gene encodes a transcription factor having inducer-binding domain and the so-called transactivating domain binding to DNA. The second gene encodes the therapeutic protein and remains under the control of a promoter able to bind the chimeric transcription factor with the inducer bound. Practically, there are two popular transcriptional activation systems: one taking advanatage of the tetracycline operon *(tet)* induced by suitable antibiotics and another, induced with the help of estrogens. Both systems have successfully been used to regulate secretion of a therapeutic protein (in this case erythropoietin) from microencapsulated cells (Serguera *et al.*, 1999).

Cancer therapy based on the use of genetically-engineered producer cells secreting in a regulated manner sufficient amounts of therapeutic proteins appears as a very attractive solution of problems confronting possible antimetastatic strategies (see Fig. 3). The proteins in question need not be antiangiogenic; the same principle described here would also apply to proteins acting at key points of other stages of metastatic process. Only intensive research effort shall show if this genetic therapy strategy is really effective.

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