

Antitumor effects of in vivo caveolin gene delivery are associated with the inhibition of the proangiogenic and vasodilatory effects of nitric oxide

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SPECIFIC AIMS

The abundance of caveolin-1 (the structural protein of caveolae microdomains) has been reported to be low or null in many transformed cells and its reexpression to result in substantial inhibition of tumor cell growth. Caveolin has therefore been proposed to function as a tumor suppressor. Still, in tumors, caveolin is expressed unaltered in the vascular compartment and more particularly in endothelial cells where it regulates the activity of the endothelial nitric oxide synthase (eNOS). The main goal of this study was to determine whether an increase in caveolin abundance in endothelial cells lining tumor blood vessels could modulate the vascular effects of nitric oxide (NO) and thereby affect the integrity of the tumor vasculature and subsequently impact tumor growth.

PRINCIPAL FINDINGS

1. In vivo cationic lipid-based transfection of caveolin cDNA leads to the selective expression of caveolin in the tumor vasculature

We used cationic lipids that are effective at targeting tumor vs. normal vascular networks to deliver plasmids to the tumor endothelium; this was validated in our experimental model by using green fluorescent protein (GFP)-encoding plasmid. Injection of the caveolin cDNA lipocomplex through the tail vein of tumor-bearing mice led to the selective expression of recombinant caveolin in the tumor vasculature. We found that when eNOS was immunoprecipitated from total lysates of caveolin-transfected tumors, increased amounts of caveolin (monomeric and oligomeric forms) could be found in the immunoprecipitate (vs. sham transfected animals; $P < 0.01$, $n = 3$). We verified by immunohistochemical analysis that eNOS was only expressed in the endothelial cells lining tumor blood vessels. When either eNOS or caveolin immunoprecipi-

tation experiments were performed from lysates of various organs of caveolin-transfected mice (including lung, liver, and spleen), we failed to detect any increase in the abundance of caveolin.

2. Recombinant caveolin expression affects tumor growth through a decrease in tumor microvessel density

Figure 1 shows the effects of the recombinant expression of caveolin on tumor growth and vascular development. Dramatic effects on tumor growth were observed in caveolin-transfected tumors as early as 2 days after transfection. While tumor diameter increased by 50% (~2mm) in 6 days in sham-transfected animals, tumor growth was almost abolished until day 8 by caveolin transfection (Fig. 1A). Several mice were killed at different times after transfection in order to evaluate potential changes in tumor microvascular density (using CD31 as an endothelial cell marker). Figure 1B shows representative histochemical pictures of the central core of the tumor collected 10 days after sham and caveolin transfection. In caveolin-transfected condition, necrosis was clearly identifiable and the areas of tumor cell survival were restricted to the direct environment of the few spared CD31-labeled structures. The evaluation of the overall vascular density revealed that caveolin transfection dramatically affected the development of the tumor vasculature between days 4 and 10 (Fig. 1C). These results were confirmed in in vitro experiments where we found that two major steps in angiogenesis (i.e., endothelial cell migration and tube organization) were blocked by caveolin transfection.

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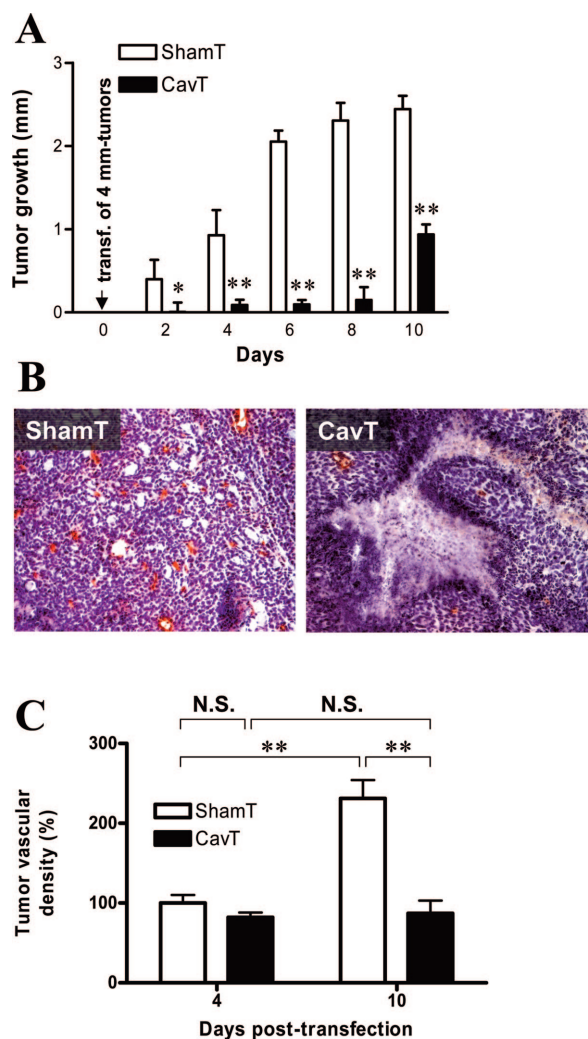


Figure 1. Caveolin-1 transfection delays tumor growth and decreases tumor vessel density. *A*) Growth of TLT tumors was determined by measuring tumor diameters in sham (open bars) and caveolin-transfected (cavT) mice (black bars). Shown is the increase in tumor diameter (over 4 mm) after caveolin/sham transfection; * $P < 0.05$, ** $P < 0.01$ vs. corresponding sham condition value ($n=10$). *B*) Representative pictures of slices of the central core of the tumors collected 10 days after sham or caveolin transfection and immunostained with antibodies directed against the EC marker CD31. *C*) Bar graph depicting the overall tumor microvessel density (integrating central and peripheral tumor areas) determined at days 4 and 10 after caveolin/sham transfection; ** $P < 0.01$ vs. corresponding sham condition value ($n=10$).

3. Recombinant caveolin expression in the tumor vasculature alters tumor NO-dependent blood flow

Although we did not find any significant difference in vascular density at day 4 post-transfection between sham- and caveolin-transfected tumors, an effect of recombinant caveolin expression on tumor growth was already detectable (compare Fig. 1A and C at day 4). To account for the early effects of recombinant caveolin expression, we reasoned that the local increase in caveolin abundance could have led to other effects on the NO signaling pathway without altering the integrity/

viability of the targeted endothelial cells. We verified that the increased interaction of eNOS with caveolin inside the tumors of transfected mice (as authenticated by coimmunoprecipitation at day 4) led to an inhibition of eNOS activity. Accordingly, the cGMP content of tumors, used as an index of NO release, was decreased by a factor of 3.6 ($P < 0.01$ vs. shamT tumors, $n=3$).

Since the eNOS/caveolin inhibitory interaction was increased in transfected tumors and did not directly lead to antiangiogenic effects (endothelial cell death), we examined whether the early inhibition in NO production could translate in changes in tumor blood flow. We first used laser Doppler imaging, which, despite limitations in resolution that complicate observations of acute changes in blood flow, is particularly well suited to track the progressive increase in global tumor blood flow day after day. **Figure 2** shows that tumor perfusion was significantly decreased in tumors as early as 4 and 6 days after caveolin transfection.

To further characterize the deficit in NO-mediated regulation of vascular tone, we also used laser Doppler microprobes to locally measure the ability of carbachol, an agonist known to stimulate eNOS activation, to induce a vasoresponse. We found that carbachol could

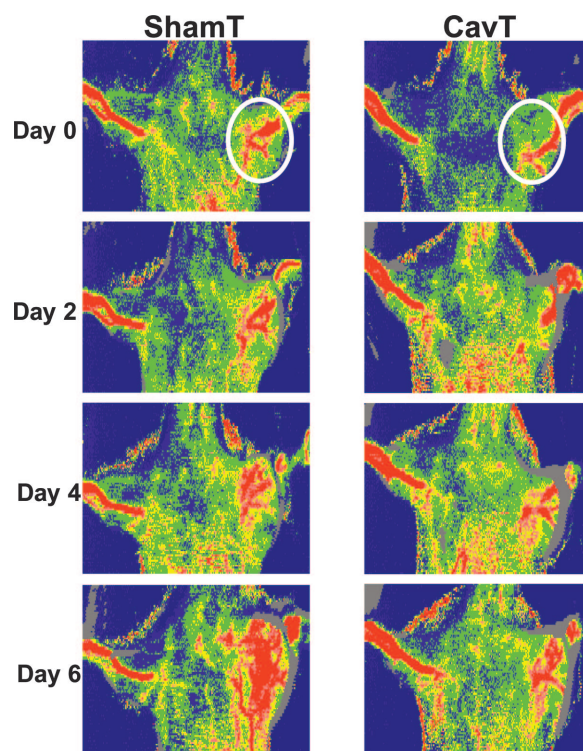


Figure 2. Caveolin transfection leads to an early decrease in tumor blood flow through an alteration in NO signaling. Shown are typical laser Doppler imaging (LDI) pictures from TLT tumor-bearing mice (see circle) at days 0, 2, 4, and 6 after sham or caveolin transfection. The perfusions of the tumor-bearing leg and of the control leg can be evaluated on the basis of the colored histogram pixels. The slow increase in tumor blood flow in caveolin-transfected mice and the rapidly expanding tumor vascularization in untreated mice are easily detectable.

actually stimulate tumor blood flow in sham-transfected tumors ($51 \pm 5\%$, $P < 0.01$, $n = 4$), whereas the agonist administration failed to induce any significant effect in caveolin-transfected mice. The NOS inhibitor L-NAME prevented the increase in carbachol-induced perfusion in sham-transfected tumor-bearing mice.

CONCLUSIONS AND SIGNIFICANCE

Although caveolin has been proposed to act as a tumor suppressor, the fact that caveolin-deficient mice do not spontaneously develop tumors has recently shaken this hypothesis. Therapeutic exploitation of the reexpression of caveolin in tumor cells (when the tumor is already developed) appears unrealistic since a very large majority of tumor cells should be transfected to lead to relevant *in vivo* antitumor effects. Here, we identified the presence of caveolin in the vascular compartment of tumors to be a very likely confounding factor in the understanding of the multiple roles of this protein in tumors, and we documented that altering its abundance in tumor endothelial cells was therapeutically relevant.

We showed that caveolin transgene (when selectively delivered by cationic lipids to tumor endothelial cells) dramatically delayed tumor development (**Fig. 3**). NO-mediated tumor angiogenesis and vasodilation were found impaired in caveolin-transfected mice. The effects on tumor blood flow were the first detectable indications that caveolin overexpression (before inducing endothelial cell death) modified intrinsic properties of the endothelium (i.e., the ability to release NO and thereby maintain tumor vessel dilation). The consequent reduction in tumor accessibility for nutrients and oxygen is very likely to account for the rapid inhibition of tumor growth. Still, caveolin transfection resulted in direct antiangiogenic effects, as validated by the reduction in vascular density and the associated necrosis in the center of the tumor.

Our study reveals that by exploiting the exquisite

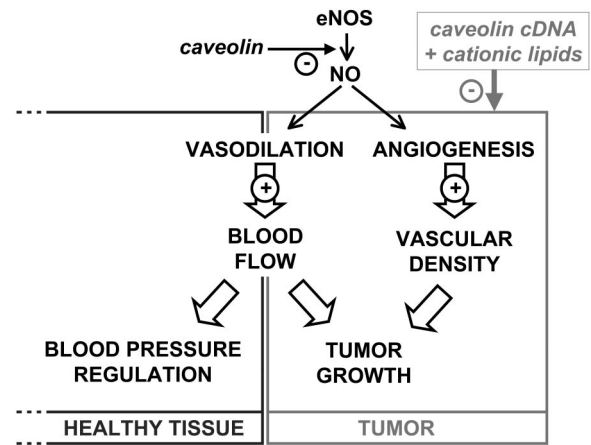


Figure 3. Schematic diagram. Major NO-mediated signaling pathways in tumors (right) and healthy surrounding tissue (left). While in adult organisms, NO-mediated angiogenesis is exclusively observed in pathological states such as during tumor development (although there are a few exceptions such as the healing and menstruation processes), NO can regulate blood flow both in healthy organs and diseased tissues, including tumors. Overexpressing caveolin, a negative allosteric regulator of the endothelial nitric oxide synthase (eNOS), may exert direct inhibitory effects on both NO-mediated processes. To maximally exploit the beneficial effects of reducing NO production in tumors (to limit the effect of altering blood flow in healthy tissues), caveolin cDNA needs to be selectively delivered to the endothelium lining tumor blood vessels. The high tropism of cationic lipids for the tumor endothelium can fulfill this need. The caveolin overexpression in the tumor vascular compartment first reduces tumor perfusion and limits the amounts of nutrients and oxygen available for the tumor growth, then exerts direct antiangiogenic effects leading to a reduction in tumor vascular density and subsequent reduction in tumor progression.

regulatory interaction between eNOS and caveolin and the propensity of cationic lipids to target EC lining tumor blood vessels, caveolin plasmid delivery appears to be a safe and efficient way to block neo-angiogenesis and vascular function in solid tumors, and thereby to affect tumor growth independent of any direct effects on tumor cells. FJ