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ARTICLE

$\alpha_v\beta_3$ Integrin-Mediated Adenoviral Transfer of Interleukin-12 at the Periphery of Hepatic Colon Cancer Metastases Induces VCAM-1 Expression and T-Cell Recruitment

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We previously reported that systemic injection of recombinant adenovirus resulted in a rim of gene transduction around experimental liver tumor nodules. This zone of higher infection is dependent on the $\alpha_v\beta_3$ integrin, acting as an adenovirus internalization receptor, which is overexpressed in tissues surrounding liver metastases. When a recombinant adenovirus encoding interleukin-12 (AdCMVIL-12) is given into a subcutaneous tumor nodule in mice also bearing concomitant liver tumors, a fraction of AdCMVIL-12 reaches the systemic circulation and infects liver tissue, especially at the malignant/healthy tissue interface. As a result of the expression at this location of the interleukin-12 transgenes, VCAM-1 is induced on vessel cells and mediates the recruitment of adoptively transferred anti-tumor cytolytic T-lymphocytes. These studies provide mechanistic explanations for the potent therapeutic synergy observed between interleukin-12 gene transfer and adoptive T-cell therapy.

Key Words: **gene therapy; $\alpha_v\beta_3$ (CD51/CD61); adenovirus; VCAM-1 (CD106); interleukin-12; adoptive T-cell therapy; immunotherapy; colon cancer.**

INTRODUCTION

Recombinant adenoviruses (1) have shown good efficacy as vehicles for immunotherapeutic genes upon intratumoral injection. In this setting IL-12 (2–5), CD40L (6, 7), IL-2 (8, 9), and GM-CSF (10) display tumor-eradicating efficacy in various rodent models. Unless artificially targeted (11), adenovirus relies on the interaction of the fiber–knob protein and the penton base protein of its capsid with cellular surface receptors for cell entry (12). Although other receptors may exist, coxsackie adenovirus receptor and the $\alpha_v\beta_3$ integrin (CD51/CD61) are the best characterized receptors for adenoviral infection (13). The $\alpha_v\beta_3$ integrin is also a well-known receptor for an RGD motif present in a number of extracellular matrix proteins mainly expressed during tissue repair (14). Although $\alpha_v\beta_3$ has a broad tissue distribution it is selectively expressed on angiogenic and not on resting endothelium (14, 15). In fact, anti-$\alpha_v\beta_3$ blocking mAbs or peptide antagonists downregulate angiogenesis with remarkable anti-tumor effects (14, 16, 17).

Intratumoral injections of recombinant adenovirus encoding both chains of IL-12 have shown intense anti-tumor effects (2–5) that can be exploited in very efficacious combinations with other transgenes such as IL-2 (8, 9), IP-10 (18), lymphotactin (9), CD80 (19), and 4-1BBL (20, 21). In most cases the anti-tumor activity needs the action of cytolytic T-lymphocytes (CTLs) (5, 22, 23), but other mechanisms such as natural killer cell activation (18, 22, 24) and angiogenesis inhibition (25, 26) are also important.

We have shown that IL-12 gene transfer synergizes with adoptive T-cell therapy not only by simplifying the obtainment of CTL cultures but also by rendering liver metastases more sensitive to an immune attack (23). It was documented that recombinant adenovirus injected into a subcutaneous tumor nodule or intravenously showed a preference for gene transduction around liver metastases, depicting a rim at the healthy/malignant borders (23). The synergy of adoptive T-cell therapy and IL-12 gene transfer at the effector phase could be explained if some-

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how IL-12 could condition the liver metastasis for lymphocyte infiltration. It has been recently reported that VCAM-1 (CD106) and ICAM-1 (CD54) are upregulated in the vessels of murine ovarian cancers when recombinant IL-12 is given systemically and its expression is induced by cytokines such as TNFα, IFNγ, and IL-1 (27–29). VCAM-1 is an endothelial counterreceptor for the VLA-4 integrin (30), which is expressed in a molecular conformation with high avidity for VCAM-1 on activated T-cells (31) and which mediates lymphocyte diapedesis (32).

In this study we report that adenoviruses preferentially transfer genes to the area limiting liver metastases because of selective expression at this place of $\alpha_v\beta_3$. By virtue of adenovirally encoded IL-12 expression at this location, a cytokine cascade that enhances VCAM-1 expression and subsequent infiltration by anti-tumor lymphocytes is elicited.

MATERIALS AND METHODS

Mice and cells. Five- to eight-week-old BALB/c female mice were purchased from Harlan (Barcelona, Spain). The 293 cell line was obtained through American Type Culture Collection (Manassas, VA). CT26 tumor cell line is an undifferentiated murine colorectal adenocarcinoma (5, 23). The hemangioma-derived murine endothelial cell line Py-4-1 from SV40 T transgenic mice was a gift from Dr. V. Baucht (University of North Carolina, Chapel Hill, NC) (33). Py-4-1 and CT26 cells were maintained in DMEM containing 10% FCS. Cell culture reagents were from Life Technologies (Basel, Switzerland).

X-gal staining and immunohistochemistry. For X-gal staining and immunohistochemistry, tissue samples were embedded in OCT compound (Tissue Tek, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. Tissue sections of 6 μm thickness or adherent Py-4-1 cells in 24-well plates were fixed with glutaraldehyde (0.5%) and stained with 5-bromo-4-chloro-3-indoyl-β-galactosidase (X-gal) as previously described (5, 23). Cryosections for immunohistochemical staining were warmed at room temperature, air dried, and fixed in prechilled acetone for 10 min. After a rinse in PBS, endogenous peroxidase activity was neutralized using DAKO peroxidase blocking reagent (DAKO Co., Glostrup, Denmark). Sections were incubated with the corresponding mAbs (anti VCAM-1, anti-CD31, anti-CD51 purchased from Pharmingen, San Diego, CA) and visualized with anti-rat IgG conjugate peroxidase (Sigma, St. Louis, MO) and the chromogenic substrate DAB (DAKO). Mayer's hematoxylin counterstaining was used.

Adenovirus construction. Recombinant adenoviruses carrying the p40 and p35 genes of IL-12 (AdCMVIL-12) or β-galactosidase (AdCMVlacZ) under the control of the CMV promoter have been previously described (5, 23). Adenoviruses were purified by double cesium chloride ultracentrifugation and extensively dialyzed against 10 mM Tris/1 mM $MgCl_2$ to be stored at −80°C in the presence of 10% (v/v) glycerol.

In vivo experiments. BALB/c mice were injected subcutaneously in the right hind flank with 5×10^5 CT26 cells in 50 μl of PBS. In some experiments, mice additionally received an identical dose of tumor cells surgically injected in the middle lobe of the liver under general anesthesia (5, 23). For treatment, subcutaneous tumors were injected with 10^9 pfu of recombinant adenovirus in 50 μl of PBS. Short-term or long-term CTL lines in PBS were injected iv. Tumor size (mean diameter) was assessed by laparotomy using a precision caliper. Anti-mouse α_v (CD51), anti-mouse VCAM-1 (CD106), and anti-mouse ICAM-1 (CD54) (no azide/low endotoxin grade mAbs) for blocking *in vivo* experiments were purchased from Pharmingen–Becton Dickinson (San Diego, CA). Statistic significance of the difference among groups was evaluated by Mann–Whitney *U* tests. IL-12 (p70) levels were measured by a sandwich ELISA from Biosource (Camarillo, CA) according to the manufacturer's instructions. Tumor tissue homogenates were carried out by Ultraturrax (IKA-WERKE Gmbh & Co., Staufen, Germany) in PBS, 100 μM PMSF, and 10 μg/ml aprotinin at 4°C. Total protein content was assessed by BCA protein assay kit (Pierce, Rockford, IL).

CTL generation and long-term anti-CT26 CTLs. Mice carrying bilateral 5- to 8-mm (diameter) subcutaneous CT26 tumors were treated with intratumoral injections of 10^8 pfu of AdCMVIL-12 each tumor. Draining lymph nodes were removed aseptically 5 days later and single-cell suspensions were obtained. Lymph node cells were cultured in 24-well plates (Greiner Labortecknik, Nurtingen, Germany) for 7 days at 5×10^6 cells/well with 2×10^5 CT26 tumor cells/well (5000 rads). Culture medium was RPMI 1640, 10% FCS supplemented on day 5 with mIL-2 (8–10 IU/ml) (Peprotech, London, UK). For long-term CTL cultures, such cells were restimulated every 10 to 14 days with 2×10^5 irradiated CT26 cells and irradiated syngeneic splenocytes plus 20 IU/ml rIL-2 (34). Such cells displayed high levels of cytotoxicity at 1:1 target:effector ratio.

Immunofluorescence and FACS analysis. Analysis of immunofluorescence staining with fluorochrome-tagged anti-VCAM-1, anti-ICAM-1, and anti-CD31 mAbs (Pharmingen) was performed on electronically gated Py-4-1 cells that were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Long-term CTL were surface-labeled with fluorescein with the PKH2-GL kit according to the manufacturer's instructions (Sigma). Fluorescent cells were examined in tissue sections under a conventional fluorescence microscope (Nikon).

Adhesion experiments. Py-4-1 cells were plated at 10^4 cells/well onto 96-well plates (Greiner Labortecknik) and cultured in RPMI 1640 medium to subconfluence. Before the experiment, Py-4-1 cells were incubated for 2 h in RPMI 1640 medium with 5 μg/ml of the corresponding mAbs that were present through the assay in triplicate wells. CTLs were labeled with ^{51}Cr (100 μCi/10^6 cells), extensively washed, and added to each well (10^5 cells/well). The plates were incubated for 2 h at 37°C and then homogeneously and gently washed four times with medium. After washing, cells in each well were lysed with 200 μl of 0.5% Triton X-100 (Sigma) and γ-radioactivity was measured in a γ counter. Percentage of CTL adhesion was calculated using the following formula: % = 100 × (experimental cpm − spontaneous cpm/maximum cpm − spontaneous cpm) as described (35). Statistical significance of the differences among groups was evaluated by Mann–Whitney *U* tests.

RESULTS

$\alpha_v\beta_3$ Mediates Selective Adenoviral Gene Transduction around Liver Metastases

BALB/c mice carrying both liver metastasis of CT26 colon carcinoma and a subcutaneous tumor nodule were injected intratumorally at the subcutaneous site with 10^9 pfu of a recombinant adenovirus encoding β-galactosidase (AdCMVLacZ). Part of the adenovirus reaches systemic circulation and infects liver tissue, giving rise to a scattered pattern of β-gal$^+$ hepatocytes (23) (Fig. 1A). Interestingly, the immediate contact area between those model live metastases and surrounding liver tissue is transduced much more efficiently (Fig. 1A). Previous studies in rabbit tumors have shown that this area is rich in expression of $\alpha_v\beta_3$ integrin in relation with angiogenic processes (15). As shown in Fig. 1B, we confirm such observations with the detection of overexpression of the β_3 chain by immunohistochemistry in tissue surrounding liver metastases, often related to vascular structures with lumen (not shown). It should be noted that β_3 can reach the plasma membrane only when associated to α_v (14), indicating that α_v must be present in the dimers with β_3.

$\alpha_v\beta_3$ is a known receptor for the penton base protein of

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adenoviral capsids (12, 13). Therefore our hypothesis was that recombinant adenovirus could be transducing more efficiently at this location precisely because of expression of this $\alpha_v\beta_3$ integrin. In order to test this hypothesis, a commercial anti-α_v mAb that blocks receptor/ligand interactions was used. First, it was demonstrated that such anti α_v antibody inhibited *in vitro* gene transduction by AdCMVLacZ into the Py-4-1 murine endothelioma cell line, which brightly expresses α_v (Fig. 2A). In contrast, levels of gene transduction were unaffected by control mAbs such as anti-CD31, which also binds to the cell surface of Py-4-1 cells. Next it was addressed whether $\alpha_v\beta_3$ was involved in the induction of the β-gal$^+$ mantle around CT26 liver metastases. To do so, intratumoral injections of low doses (10^7 pfu) of AdCMVLacZ were performed. Under such conditions the β-gal$^+$ rim could be observed in five of six identically treated mice (Fig. 2B). However, if the adenovirus was co-injected intratumorally with 50 μg of anti-α_v mAb, the rim was undetectable in four of five cases (Fig. 2C) by two observers blinded to the conditions of the experiments. In this case peritumoral rim formation was induced with intratumoral instead of systemic AdCMVLacZ injection to permit co-injection with mAbs, thus achieving high local concentrations of the antibody in the liver tumor. Similar quantities of polyclonal rat IgG failed to inhibit rim formation (not shown). These findings clearly indicate that $\alpha_v\beta_3$ is involved in the selectivity of this area to gene transfer by adenovirus.

Similar experiments were also carried out using AdCMVIL-12 instead of the adenovirus coding for a reporter gene. For these experiments large liver CT26 tumors were chosen (diameter >10 mm) and in these cases 10^8 pfu of AdCMVIL-12 given intratumorally could stabilize tumor sizes as assessed 11 days later by surgical inspection (Fig. 2D). In this setting, as a result of co-injection of 50 μg of the anti-α_v mAb with AdCMVIL-12, the anti-tumor effect is lost since the tumors progressed, doubling their sizes (Fig. 2D), as they do in untreated animals (not shown). Therefore gene transduction by AdCMVIL-12 to achieve clinical efficacy is critically dependent on $\alpha_v\beta_3$.

To rule out that anti-α_v mAbs were blocking adenovirus-mediated IL-12 gene transfer at the subcutaneous tumor nodule, experiments were performed in which mice received 50 μg iv of anti-α_v or of control rat IgG. Under such conditions systemic biodistribution of the whole dose of the antibodies is achieved. Even under these conditions adenovirus-mediated gene transduction of IL-12 into the subcutaneous tumor nodule was not inhibited, as assessed by IL-12 (p70) relative protein concentration measured by ELISA on tissue homogenates taken 72 h after intratumor injection of AdCMVIL-12 (Fig. 3). Moreover, in experiments performed identically to those shown in Fig. 2C levels of IL-12 at the subcutaneous tumor site were similar despite treatment with 50 μg of anti-α_v mAb injected at the liver tumor nodule (data not shown).

IL-12 Gene Transduction around Liver Metastases Induces VCAM-1 Expression on Vessel Cells

When BALB/c mice carrying CT26 liver metastasis and a subcutaneous tumor nodule were given 10^9 pfu of AdCMVIL-12 at the subcutaneous site, we observed that VCAM-1 was induced on the surface of vessels inside, but mainly surrounding, liver malignant tissue. Such results were obtained by immunohistochemistry in five of five mice and a representative case is shown in Fig. 4A. As was confirmed by analysis of serial sections with anti-CD31 mAbs (data not shown), VCAM-1 is expressed on endothelial cells. In contrast, similar mice treated with identical doses of AdCMVLacZ did not show VCAM-1 expression at those locations (Fig 4B), offering a proof for the involvement of IL-12. There is no published indication that endothelium expresses receptors for IL-12 and therefore a downstream cytokine cascade set in motion by IL-12 is the likely mediator for VCAM-1 upregulation and maybe for other proinflammatory changes on endothelial cells.

IL-12-Dependent VCAM-1 Expression on Endothelium Is Required for the Efficacy of Adoptive T-Cell Therapy

Using long-term CTL lines specific for CT26 (23), we found basal *in vitro* adhesion of such lymphocyte cultures to Py-4-1 endothelial cells that brightly express VCAM-1 and ICAM-1 (Fig. 5A). The percentage of adhering lymphocytes was inhibited either by anti-VCAM-1 or by anti-ICAM-1 mAbs to approximately one-third of basal attachment, as assessed in conventional adhesion experiments (Fig. 5B). Interestingly, no further inhibition was observed with a combination of anti-ICAM-1 and anti-VCAM-1 mAbs.

To ascertain as to whether VCAM-1 induction *in vivo* by AdCMVIL-12 was important for its synergy with adoptive T-cell therapy, we treated mice bearing a hepatic and a subcutaneous CT26 tumor on day 7 after tumor inoculation with 10^9 pfu of AdCMVIL-12 inside the subcutaneous site and with 5×10^6 anti CT26 CTLs iv on day 10 after tumor engraftment. It was observed that this regime caused complete regressions in three of five mice and that the remaining liver tumors were smaller than those found in control untreated mice (Fig. 5C). In contrast, if anti-VCAM-1 mAb was repeatedly given intravenously to otherwise identically treated mice the anti-tumor effects of the CTL + AdCMVIL-12 combination were abrogated (Fig. 5C). The subcutaneous tumor nodules in these VCAM-1-blocked mice also progressed, indicating that this was not an exclusive property of the liver tumors (not shown). Inhibition of tumor rejection has been also observed upon anti-VCAM-1 systemic treatment in mice bearing only subcutaneous CT26 tumors that were treated intratumorally with 10^9 pfu of AdCMVIL-12 (Mazzolini *et al.*, manuscript in preparation), indicating that VCAM-1 upregulation was important even in the absence of CTL adoptive transfer as has been previously demonstrated by

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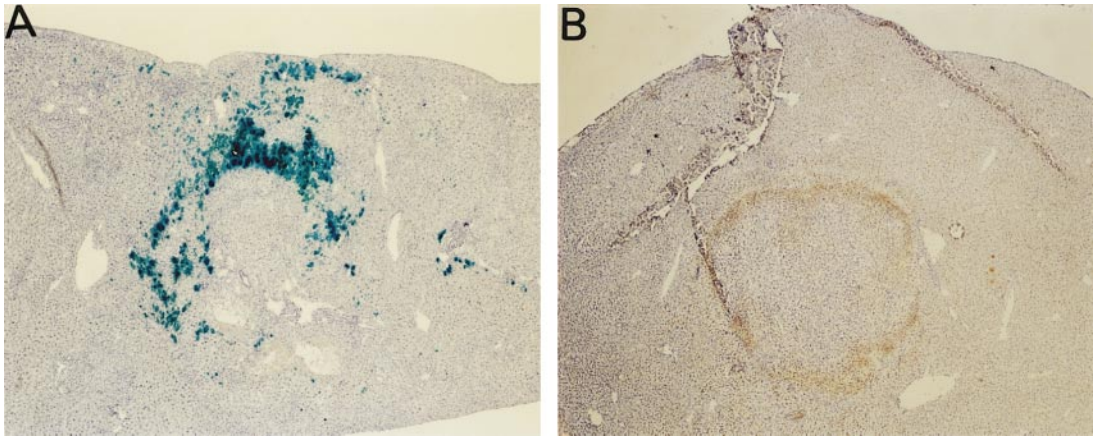

FIG. 1. $\alpha_v\beta_3$ integrin colocalizes in peritumoral areas of intense gene transduction with adenovirus. (A) Mice bearing two CT26 tumors, one in the liver and one subcutaneous, were treated at the subcutaneous site with 10^9 pfu of AdCMVLacZ. 48 h later liver sections were stained with X-gal (original magnification $\times$100). (B) Immunohistochemical staining with anti β_3 mAb of livers from mice with tumors induced by injection of CT26 tumor cells 8 days before the organs were harvested (original magnification $\times$100).

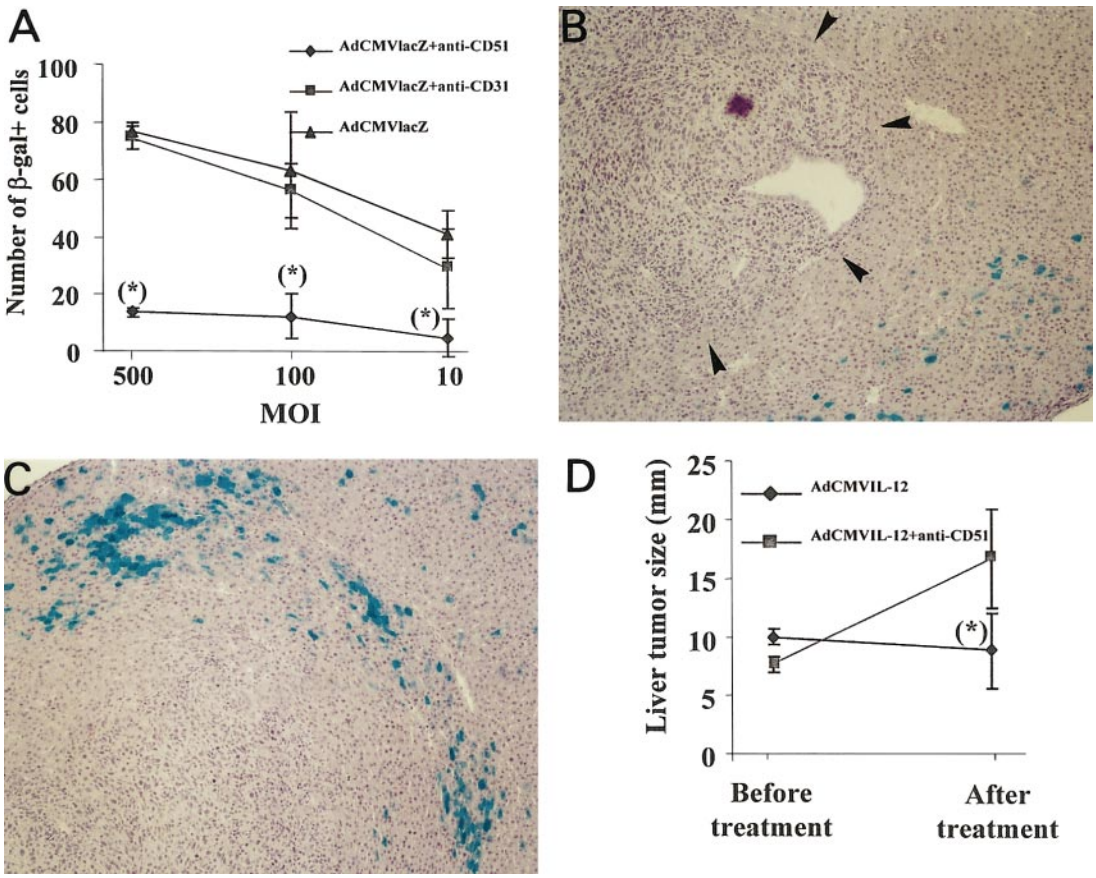

FIG. 2. Selective gene transduction with adenovirus around liver metastases is dependent on $\alpha_v\beta_3$ expression. (A) Py-4-1 cells were infected at the indicated m.o.i.'s with AdCMVLacZ in the presence of 10 μg/ml of the indicated antibodies. Results (means $\pm$ SEM) are from three independent experiments. (B, C) Mice bearing CT26 liver tumors were intratumorally injected with 10^7 pfu of AdCMVLacZ with (B) or without (C) 50 μg of anti-α_v mAb. 48 h later liver cryosections were studied by X-gal staining (original magnification 200$\times$). Arrowheads point at the tumor/healthy tissue limit. (D) Mice bearing CT26 liver tumors were injected intratumorally with 10^8 pfu of AdCMVIL-12 with or without 50 μg of anti α_v mAb. Statistical significance ($P = 0.05$) was assessed by Mann–Whitney *U* test.

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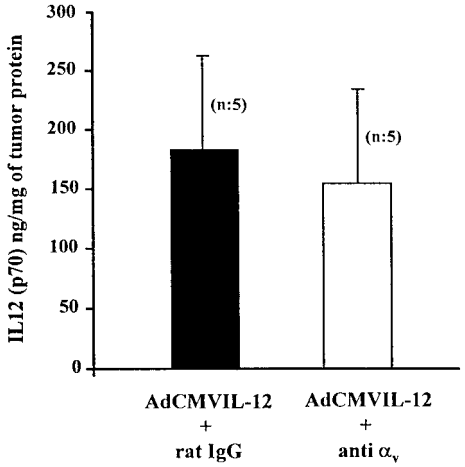

FIG. 3. Systemic treatment with 50 μg of anti-α_v mAb does not interfere with AdCMVIL-12 transduction at the sc tumor nodule. Mice carrying 5–7 mm sc CT26-derived tumors received 50 μg of anti-α_v or rat IgG (control) as indicated. Subsequently 10^8 pfu of AdCMVIL-12 was given inside the tumor nodule. 72 h later tumors were excised and homogenized to measure IL-12 (p70). Results are presented as means ± SEM.

others using recombinant proteins instead of gene transfer (27, 28).

The most likely explanation for these results is that anti-VCAM-1 mAbs are interfering with the entrance into malignant tissue of anti-tumor CTL. In order to provide a proof for this idea, we performed experiments in which adoptively transferred T-cells were labeled with fluorescein. In these experiments mice hosting 7-day-old CT26 liver tumors were injected intravenously with AdCMVIL-12 at low doses (5×10^7 pfu) to mimic systemic leakage of the adenovirus from an intratumorally injected subcutaneous nodule. Then mice were adoptively transferred iv with 2×10^6 long-term passaged anti-fluorescein-tagged CT26 CTLs. As shown in Fig. 6A, hepatic tumors were infiltrated 24 h later by high numbers of fluorescent cells that were markedly decreased in the case of mice systemically pretreated with anti-VCAM-1 mAb (Fig. 6B). These results were quantified on fluorescent cells per microscopic field basis in two experiments with four mice each (Fig. 6C). Our data indicate that VCAM-1 is critically required for tumor infiltration by CTLs.

DISCUSSION

Previous work from our group had shown that gene transfer of tumor masses with AdCMVIL-12 resulted in upregulation of CTL activity (5), simplifying the obtainment of CTL cultures for adoptive T-cell therapy (23). These CTL lines when adoptively transferred were not efficacious against large liver metastases, but if a simultaneously implanted subcutaneous tumor was treated with AdCMVIL-12, then there were remarkable synergistic effects against the liver tumors (23).

How is this taking place? We have observed that some adenovirus escapes the subcutaneous malignant tissue and presumably through systemic circulation reaches the liver toward which adenoviruses show tropism (23). Interestingly the level of gene transduction was far more intense in the immediate neighborhood of the liver metastases. When dealing with models of colorectal cancer, this is considered an important finding in itself because the liver is a frequent and regularly fatal localization for colon carcinomas.

The $\alpha_v\beta_3$ integrin has been identified both as an adenovirus coreceptor (13) and as a marker of angiogenic endothelium (14), known to be expressed in the vicinity of tumor tissue (15). Putting together both pieces of information, experiments were designed to confirm that the special adenoviral tropism for this area was due to $\alpha_v\beta_3$/adenoviral interactions. The attachment of $\alpha_v\beta_3$ with its ligands can be inhibited by specific mAbs that in our hands also block transduction of a CD51 endothelioma cell line *in vitro*. The blocking mAb could also prevent *in vivo* the formation of the peritumoral mantle of gene transduction, at least when the mAb is co-injected in the

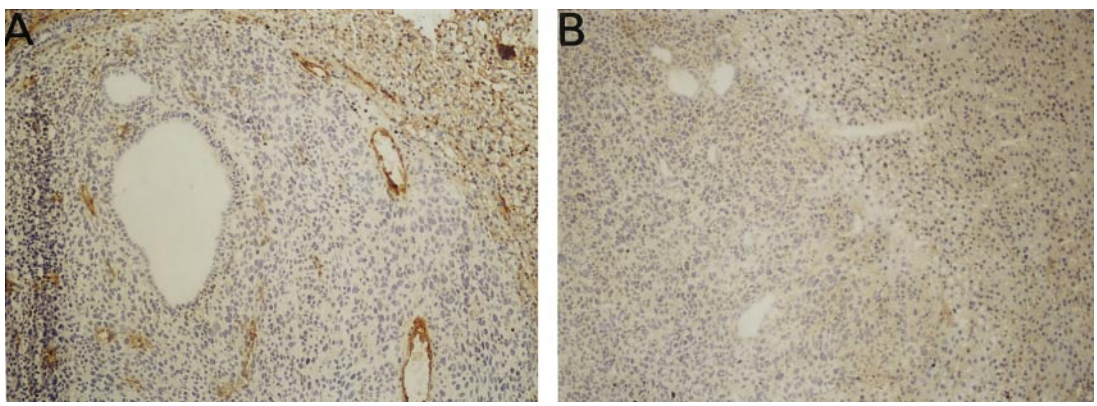

FIG. 4. AdCMVIL-12 induces VCAM-1 on tumor endothelium. Immunohistochemical staining of VCAM-1 in live tissue from mice bearing two CT26-derived tumors, one in the liver and one subcutaneous in the right flank, that were injected intratumorally at the subcutaneous site with 10^8 pfu of AdCMVIL-12 (A) or AdCMVLacZ (B). Microscopic fields (×200) are representative of multiple observations in at least five livers and identity of endothelial cells was confirmed with anti-CD31 staining in serial sections (not shown).

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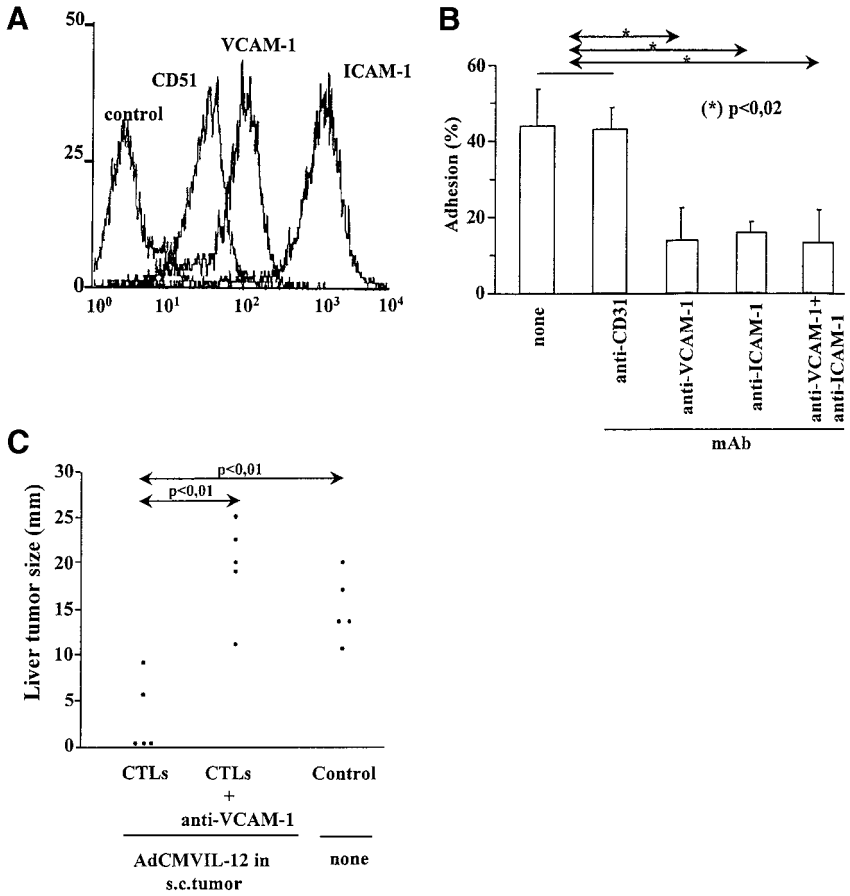

FIG. 5. Anti-VCAM-1 mAb blocks *in vitro* adhesion of anti-CT26 CTL cultures to Py-4-1 endothelioma cells and abrogates the synergy between IL-12 gene transfer and adoptive T-cell therapy. (A) FACS analysis of the expression of VCAM-1 (CD106), ICAM-1 (CD54), and CD31 on the surface of Py-4-1 cells. (B) Short-term anti-CT26 CTL cultures, obtained by coculture of lymph node cells of mice rejecting CT26 sc tumors (upon intratumoral treatment with AdCMVIL-12) with irradiated CT26 cells, were labeled with ^{51}Cr and analyzed for adhesion to subconfluent monolayers of Py-4-1 cells in the presence of the 5 μg/ml of the indicated mAb. Data are the means and SD of the three independent experiments. (C) Mice bearing two CT26 tumors nodules (5–7 mm in diameter), one subcutaneous and one in the liver, were injected with 10^9 pfu of AdCMVIL-12 in the subcutaneous tumor and with 5×10^6 iv anti-CT26 CTLs (as those in B) 3 days later. Thereafter mice received low ip doses of IL-2 every other day for 1 week. Some mice (as indicated) also received two iv doses of 250 μg/dose of anti-VCAM-1 mAb on the same day of T-cell injection and 2 days later. Size of the liver tumor nodules was assessed surgically 11 days after treatment onset. Statistic significance of the difference among groups was evaluated by Mann–Whitney *U* tests.

same bolus with the adenovirus in order to enforce competition for the binding site under conditions of antibody molar excess.

Ligation of $\alpha_v\beta_3$ in tumor vasculature by mAb or by peptides has powerful anti-tumor effects related to angiogenesis inhibition (16, 17). Adenovirus-expressing reporter genes such as AdCMVLacZ at the doses that we use in this study seem not to significantly inhibit angiogenesis, probably because the level of ligation of $\alpha_v\beta_3$, although able to help viral entry, is not enough to saturate $\alpha_v\beta_3$ molecules. Moreover, 50 μg of blocking anti α_v, which inhibited gene transduction by AdCMVLacZ, was also insufficient to display macroscopic anti-tumor activity. There are adenoviruses in which the knob fiber has been engineered to carry $\alpha_v\beta_3$-binding peptides to be targeted more efficiently to angiogenic vessels and it has been shown that such viruses are more efficient at targeting tumors than those carrying the wild-type knob–fiber (11). It would be interesting to see if those adenoviruses also create the selective peritumoral rim of gene transfer that has been observed with conventional adenovirus.

The other major question raised in our previous studies was how AdCMVIL-12 changes the tumor nodule to render it a feasible target for adoptively transferred T-cells. A major clue came from a series of studies at the University of Osaka (27, 28, 36) in which it was reported that the anti-tumor effects of systemic doses of IL-12, as a purified recombinant protein, were related to its ability to promote the expression of adhesion molecules on endothelium of a murine ovarian carcinoma in a IFNγ-dependent fashion.

Our results with IL-12 gene transfer indicate that VCAM-1 is upregulated in tumor vasculature, therefore opening a potential entrance into malignant tissue for VLA-4^+ activated T-lymphocytes. The importance of these findings is highlighted by the fact that homing of effector immune cells into the tumor is probably the hardest obstacle for the efficacy of adoptive T-cell therapy (37, 38). In order to prove these mechanisms, experiments showed that treatment with anti-VCAM-1 blocking antibodies eliminated the anti-tumor synergy of IL-12 gene transfer and T-cell adoptive therapy. In addition, our experiments transferring fluorescence-labeled anti-tumor T-cells intravenously to mice on treatment with blocking anti-VCAM-1 mAbs clearly suggest that VCAM-1 is a necessary, if not exclusive, point of entry for immune effector CTLs to meet malignant cells. Data could be also interpreted in the sense that interactions of VLA-4/VCAM-1 are also important to provide costimulatory signals to T-cells via VLA-4 (30), beyond inhibition of homing. Although possible, this is an unlikely explanation for the inhibition of the anti-tumor effects since other more important costimulatory pathways for tumor immunity (39) should be intact under VCAM-1 blockade.

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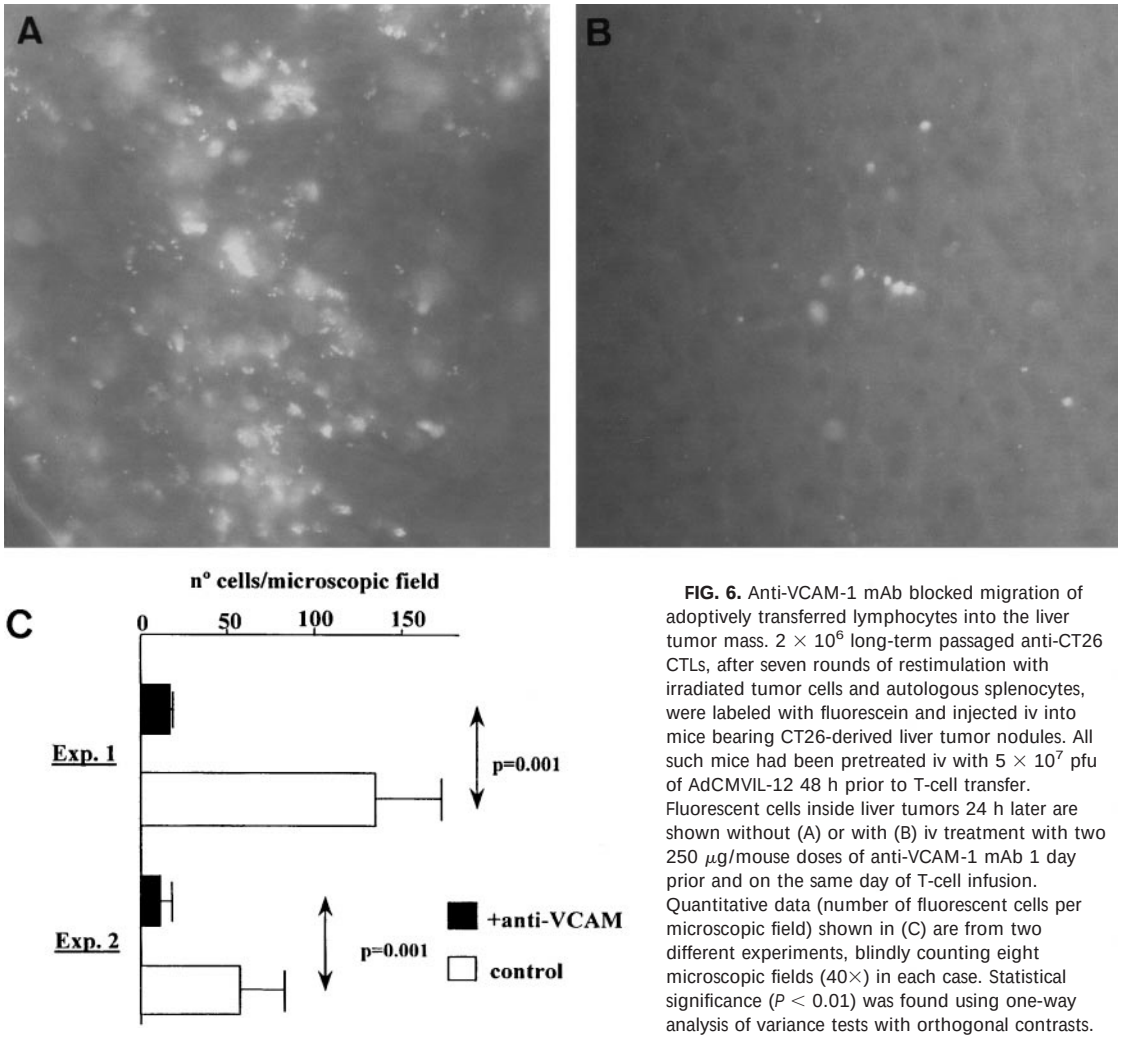

FIG. 6. Anti-VCAM-1 mAb blocked migration of adoptively transferred lymphocytes into the liver tumor mass. 2×10^6 long-term passaged anti-CT26 CTLs, after seven rounds of restimulation with irradiated tumor cells and autologous splenocytes, were labeled with fluorescein and injected iv into mice bearing CT26-derived liver tumor nodules. All such mice had been pretreated iv with 5×10^7 pfu of AdCMVIL-12 48 h prior to T-cell transfer. Fluorescent cells inside liver tumors 24 h later are shown without (A) or with (B) iv treatment with two 250 μg/mouse doses of anti-VCAM-1 mAb 1 day prior and on the same day of T-cell infusion. Quantitative data (number of fluorescent cells per microscopic field) shown in (C) are from two different experiments, blindly counting eight microscopic fields (40×) in each case. Statistical significance ($P < 0.01$) was found using one-way analysis of variance tests with orthogonal contrasts.

How VCAM-1 is being induced in tumor vasculature is not fully elucidated. Dependence on IFNγ has been proved by others (36), but a combination of cytokines elicited by IL-12 and IFNγ is probably causing the effect. A possible role for Kupffer cells and liver-resident NK-T cells as a potential source of these downstream-operating cytokines elicited by IL-12 is being currently addressed in our laboratory. Most likely, VCAM-1 is not the only proinflammatory molecule induced on endothelium with adhesion properties for leukocytes. In this regard systemic IL-12 causes upregulation of ICAM-1 as well (27). However, our data clearly indicate that VCAM-1 is critically important for the therapeutic effects of AdCMVIL-12 even in the absence of T-cell adoptive therapy, since its selective inhibition abrogates the anti-tumor effects. Confirmation of these results with gene-targeted mice rather than with blocking antibodies would be interesting but it is not possible since VLA-$4^{-/-}$ mice die at embryo stage (40) and VCAM-$1^{-/-}$ mice have not been reported yet.

Are proinflammatory changes the only ones acting on endothelium upon AdCMVIL-12 treatment? The answer is clearly no. Several reports have shown powerful antiangiogenic effects of IL-12 (25), which are mainly mediated by secondary chemokines (41–43). We have confirmed such data by implanting Matrigel plugs embedded in VEGF that get heavily vascularized unless mice are treated with AdCMVIL-12 (44). In accordance, we conclude that the synergy between AdCMVIL-12 and adoptive T-cell therapy is mediated by $\alpha_v\beta_3$-dependent gene transduction of peritumoral tissue and on the induction of proinflammatory and antiangiogenic changes in peritumoral vascular endothelium.

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