CD137 (ILA/4-1BB) is a member of the tumor necrosis factor receptor family. It was isolated from activated T lymphocytes in mice and humans. While expression of CD137 messenger RNA (mRNA) can be detected in all activated hematopoietic cells, expression of CD137 protein is specific for T lymphocytes, implying a stringent posttranscriptional control for protein expression.

Costimulation through CD137 enhances T-lymphocyte activation, comparable to costimulation through CD28, and induces rejection of tumors in mice. The CD137 ligand is expressed by antigen-presenting cells, and bidirectional signaling has been demonstrated for CD137. Cross-linking of the membrane-bound CD137 ligand inhibits T-lymphocyte proliferation and induces apoptosis. In monocytes, cross-linking of the CD137 ligand causes activation and prolongation of survival and proliferation.

Soluble forms of CD137 are generated by differential splicing and are expressed specifically by activated T lymphocytes. Their levels correlate with activation-induced cell death in T lymphocytes.

Expression of CD137 is not restricted to immune cells. CD137 mRNA has been detected in activated chondrocytes and epithelial and hepatoma cells. Expression of CD137 protein has been verified in chondrocytes.

In all primary cells, expression of CD137 mRNA and protein is strictly dependent on activation. A constitutive expression of CD137 mRNA was found only in several tumor cell lines, implying a potential growth advantage of CD137 expression for tumor cells.

This hypothesis prompted us to study whether CD137 protein also would be expressed constitutively by tumor cells in vivo. By using immunohistochemical studies, we found that CD137 can be expressed on blood vessel walls by the
endothelial cells and the vascular smooth muscle cells (VSMCs). CD137-positive vessels are not present in healthy tissues. They occasionally occur in benign tumors and inflammatory tissues but are found at a significantly higher rate in malignant tumors.

Materials and Methods

Reagents

Anti-CD137 antibody (clone BBK-2) and its isotype control, MOPC21, were obtained from Bioscource (Ratingen, Germany) and Sigma (Deisenhofen, Germany), respectively.

Tissue Samples

Grossly normal tissue was taken from a site distant from the lesion of fresh tissue after surgical removal. We studied the following 32 fresh specimens from various organs: lung and liver, 5 (16%) each; colon and soft tissue, 4 (12%) each; thyroid gland, 3 (9%); stomach, parathyroid gland, and spleen, 2 (6%) each; parotid gland, heart, aorta, kidney, and lymph node, 1 (3%) each. The mean age of the patients was 54 years (range, 15-78 years), and the male/female ratio was 2.3 (21:9).

In addition, we studied the following 61 fresh specimens from diseased tissues: gastrointestinal tract (including liver, parotid gland, gallbladder, and pancreas), 15 (25%); lymphatic system, 12 (20%); endocrine system and female genital tract, 8 (13%) each; soft tissue, 6 (10%); respiratory tract, 4 (7%); and urinary tract, male genital tract, cardiovascular system, and skin, 2 (3%) each (Table 1).

There were 14 benign tumors (23%), 34 malignant tumors (56%), 9 specimens of inflammatory tissue (15%), and 4 samples not grouped (7%). In all cases, the diagnosis was based on histologic examination.

Immunohistochemical Studies

Frozen tissue sections were fixed with 2% paraformaldehyde for 10 minutes. Endogenous peroxidases were inactivated by 2% hydrogen peroxide in methanol for 15 minutes. Unspecific staining was blocked by 3% dry milk in phosphate-buffered saline for 30 minutes. Two micrograms per milliliter of anti-CD137 (clone BBK-2) or an isotype control antibody (MOPC21) in 3% dry milk was added overnight. Positive hybridization was detected by using the ABC staining kit (Dako, Hamburg, Germany) at 37°C using diaminobenzidine as the substrate. After each step, the samples were washed 3 times with phosphate-buffered saline. Tissue sections were stained with hematoxylin and embedded in Entellan (Merck, Darmstadt, Germany).

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was isolated from peripheral blood mononuclear cells using RNAzol B (Tel-Test, Friendswood, TX), and up to 5 µg of RNA were reverse-transcribed in a 20 µL volume, using random hexanucleotide primers (50 µg/mL), a 25-µmol/L concentration of deoxynucleoside triphosphate (dNTP), a 10-mmol/L concentration of dithiothreitol, 200 U of SuperScript II RNaseH-RT (BRL, Eggenstein, Germany), and 20 U of RNAsin (Roche, Mannheim, Germany) for 60 minutes at 42°C.

Two microliters of the reverse transcriptase reaction served as template for the subsequent polymerase chain reaction, which was performed in a 20-µL volume with 1 U of Taq DNA polymerase (Roche), a 200-µmol/L concentration of dNTPs, a 1.5-mmol/L concentration of magnesium chloride, a 10-mmol/L concentration of tris(hydroxymethyl)aminomethane (pH 8.3), a 50-mmol/L concentration of potassium chloride, and a 10-µmol/L concentration of each primer. After a 5-minute denaturation step at 94°C, the reaction proceeded in 30 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 1 minute at 72°C, followed by 10 minutes at 72°C.

The primers were used as follows:

**CD137 sense:** 5’-ATCATGGGAAACAGCTTGTAAC-AAC

**CD137 antisense:** 5’-TGGTCCACAGACCAGTCCTTC

**Cyclophilin sense:** 5’-GACAAGGTCCCAAA-TGGAC

**Cyclophilin antisense:** 5’-GACAAGGTCCCAAAGACAGACG

Statistics

Significance values were calculated using the chi-square test.

Results

We undertook an immunohistochemical screening study to determine expression of CD137 in vivo under physiologic and pathologic conditions. We studied 93 human tissue samples from healthy and diseased organs (Table 1).

Strong expression of CD137 was found on blood vessel walls in 15 tissue samples. In most cases (10), the endothelial cell layer and the VSMCs stained positive for CD137. In 3 tumors, leiomyosarcoma, fibrosarcoma, and rhabdomyosarcoma, the endothelial layers were negative, while the VSMCs displayed strong staining for CD137.

CD137-expressing blood vessels could not be found in any of the 32 healthy tissue samples. Furthermore, in inflammatory tissues and in benign tumors, only 2 (22%) of 9 and 2...
(14%) of 14 tissue samples, respectively, contained CD137-positive vessels. However, 11 (32%) of 34 malignant tumors contained CD137-positive vessels, a significantly higher rate ($P < .01$).

Several methods were used to verify that the monoclonal antibody used (clone BBK-2) recognizes CD137 rather than merely cross-reacting with an unrelated antigen. First, BBK-2 was incubated with CD137-transfected COS or mock-transfected COS cells for 1 hour at room temperature before being used in immunohistochemical studies. This preabsorption on CD137-expressing cells abolished staining on a parallel section of the Leydig cell tumor sample that was used in Image 1. Second, the immunohistochemical staining was repeated on selected tissue samples with a polyclonal chicken anti-CD137 serum that yielded identical results (not shown). Third, a mixed culture of primary endothelial cells and VSMCs expressed CD137 mRNA on activation (Image 3).

No significant difference in CD137 expression could be detected between veins (10/15 positive cases) and arteries (6/15). In some tumors, all vessels stained positive for CD137, whereas in others, for example, the Leydig cell tumor, CD137-positive vessels were found interspersed with CD137-negative vessels (Image 1). Only frozen tissue samples were used since no CD137 staining could be obtained with the anti-CD137 antibody (clone BBK-2) on paraffin-embedded tissues.

**Discussion**

We studied in vivo CD137 expression in human tissue samples. No expression of CD137 could be detected in
tumor cells. This was an unexpected result, since CD137 mRNA expression was expressed constitutively in several tumor cell lines, while its expression in primary cells is strictly dependent on activation. Therefore, our original hypothesis that tumor cells may express CD137 because of a growth or selection advantage could not be confirmed.

However, strong expression of CD137 was found in blood vessel walls. Expression of CD137 in blood vessels does not seem to be dependent on the type of organ the vessels are supplying but rather on the nature of the tissue alteration. Even in the malignant tumors, and more so in the benign tumors and in inflammatory tissues, only a minority of samples contained CD137-positive vessels. Furthermore, 3 of 4 sarcomas but only 4 of 15 carcinomas contained CD137-positive vessels. This indicates that distinct physiologic conditions may be required for blood vessels to express CD137. The nature of these conditions is unknown at present.

In most cases of CD137-positive blood vessels, the endothelial cells and the VSMCs expressed CD137. Two cases in which only the endothelial cells were stained and 3 cases in which only the VSMCs were stained were found. These cases may represent intermediary stages of vessels starting to express CD137. It is noteworthy that the 3 cases with solely VSMC staining all were sarcomas.

In the present study, we found tumors in which all blood vessels expressed CD137 and others in which CD137-positive and CD137-negative vessels were found side by side. The potential significance of this observation is the focus of ongoing work in our laboratory.

The complete absence of CD137-positive vessels in healthy tissues may allow the use of CD137 as a target for an antiangiogenic tumor therapy. CD137 also is expressed on activated T lymphocytes, but levels of expression on blood vessels far exceed those on T lymphocytes. Even in inflamed...
tissue samples in which strong CD137 staining was obtained on blood vessels, CD137 could hardly be detected on infiltrating T lymphocytes. Therefore, the potential side effects of a therapy directed against CD137-expressing cells, ie, immunosuppression, may be tolerable.

Anti-CD137 antibodies have been proven to be potent antitumor agents. Injection of anti-CD137 antibodies in mastocytoma and sarcoma-bearing mice led to a complete remission of the tumors and lasting antitumor immunity. An enhancement of the activity of CD137-expressing helper and cytotoxic T lymphocytes by the anti-CD137 antibodies could be identified as an underlying mechanism. Theoretically, antibody-dependent cell cytotoxicity or complement lysis of CD137-expressing endothelial cells could have been a contributing second mechanism.

While we did not find CD137 in vessels of healthy tissue samples, Boussaud et al described the expression of CD137 in capillaries and vessels of a normal lung. However, these authors used grossly normal-looking lung tissues distant from the site of the bronchial carcinomas. We have encountered similar cases in which we found CD137-positive vessels in seemingly healthy tissue sections of cancer-infested organs. Detection of CD137-positive blood vessels therefore may provide a valuable and novel parameter for the differentiation of benign from malignant tumors.

CD137 delivers a potent costimulatory signal to T lymphocytes. Whether CD137 also can deliver a signal to blood vessel cells and the nature of this potential signal are unknown. The CD137 ligand is expressed on antigen-presenting cells. Bidirectional signal transduction has been shown for the CD137 receptor–ligand system. The signal through the CD137 ligand induces activation of and adherence by blood monocytes. Therefore, CD137 on the endothelium could cause monocyte activation.
and may contribute to monocyte extravasation into the tissue.

Activated monocytes contribute to the prolongation and enhancement of inflammatory reactions.\textsuperscript{19,20} CD137-mediated recruitment of monocytes to sites of inflammation therefore may worsen chronic inflammatory conditions. The role of monocytes and macrophages in tumor growth or rejection is ambiguous. These cells constitute a major component of the peritumoral inflammatory infiltrate and have been implicated in tumor regression by phagocytosis and by the release of cytotoxic compounds, such as reactive oxygen intermediates, nitric oxide, and arachidonic acid metabolites.\textsuperscript{21} On the other hand, activated monocytes and macrophages also release extracellular matrix–degrading enzymes such as matrix metalloproteinases, plasminogen, and plasminogen activator, which may support tumor growth and metastasis.\textsuperscript{22} It may be this latter activity that provides tumors with a growth advantage if they succeed in facilitating monocyte recruitment by inducing CD137 expression on blood vessel walls.

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References


Image 3 | Endothelial cells and vascular smooth muscle cells (10\textsuperscript{6} per condition) were cultured for 24 hours, unstimulated (control) or activated with phorbol 12-myristate 13-acetate (PMA; 5 ng/mL) + the calcium ionophore A21387 (100-nmol/L concentration). Expression of CD137 messenger RNA (A) was tested by reverse transcriptase–polymerase chain reaction with exon-spanning primers. Comparable amounts of complementary DNA (cDNA) were verified by amplification of cyclophilin (B): cDNA from lymphocytes activated by PMA (5 ng/mL) + the calcium ionophore A21387 (100-nmol/L concentration) for 24 hours served as a positive control.


