Endothelial Expression of CD40 in Renal Cell Carcinoma

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ABSTRACT

Recently, the immunoregulatory molecule CD40 has also been introduced as a potential surface determinant of endothelial cells that can be induced by various cytokines and thus might be involved in inflammatory vascular reactions. In this study, the ubiquitous endothelial expression of CD40 within the neovascularized areas of renal cell carcinoma is demonstrated. The strong capillary expression of CD40 in 12 tumor samples is contrasted by the absence of endothelial CD40 in the corresponding tumor-free kidney specimens in which only certain tubular segments and few interstitial cells carry CD40. Northern hybridization studies confirmed the presence of CD40 RNA in cytokine-treated endothelial cells and in renal cell carcinoma, whereas no hybridization signal was obtained with normal kidney tissue. That the presence of tumor cells is pertinent to the endothelial expression of CD40 could be substantiated by in vivo experiments, when a renal carcinoma cell line and its sublineant, but not normal kidney cells, could induce CD40 on endothelial cells in culture. According to further experimental results, the carcinoma-derived, CD40-inducing factor(s) is not represented within a variety of pleiotropic cytokines including IFN-γ, interleukin 1, interleukin 6, and tumor necrosis factor α, or common angiogenic factors such as basic fibroblast growth factor, vascular endothelial cell growth factor, angiogenin, and erythropoietin. The immunohistological results showing a widespread, even distribution of CD40 in tumor capillaries suggest that within renal cell carcinoma, the appearance of endothelial CD40 may also be related to angiogenesis in addition to inflammation.

INTRODUCTION

The CD40 molecule, a member of the TNF/receptor family (1), has been recognized as a cell surface molecule that is centrally engaged in the activation of B lymphocytes. In addition to the production of immunoglobulins and the switch of immunoglobulin isotypes that is promoted through CD40, CD40 also protects B cells within germinal centers against spontaneous apoptosis (2). Hence, CD40 is predominantly and physiologically expressed on immune cells of B lineage; however, malignant cells, including leukemias as well as Hodgkin’s and non-Hodgkin’s lymphomas, also carry CD40 (3). Owing to its participation in the process of antigen presentation, CD40 can further be detected on monocytes (4), Langhans cells, and follicular dendritic cells (5). It is of note that immunization of mice with urinary bladder carcinoma yielded the first mAb reactive against CD40 (6). In general, CD40 appears to be associated with the capacity of certain cell types to differentiate, proliferate, and communicate intercellular signals.

Vascular ECs are versatile cells that serve many immunological as well as nonimmunological functions. An investigation of CD40 expression on ECs therefore seems rewarding. Only a few studies have addressed this question with equivocal results so far, in that CD40 could not be found on thymic ECs (7), whereas low constitutive expression was seen on microvascular endothelia in normal skin and on cultured umbilical vein ECs (8). Cytokines such as IFN-γ or TNF-α could rapidly up-regulate CD40 on cultured ECs, and ligation of CD40 in turn led to increased expression of adhesion molecules and adherence of leucocytes (9), suggesting a potential role of CD40 in inflammatory vascular reactions.

In this study, we were interested in whether CD40 was involved in the process of neovascularization of tumors. RCCs of the clear-cell type served as an in vivo model of extensive, tumor-associated capillary proliferation. Endothelial expression of CD40 was assessed in tissue sections from RCC and was compared with the distribution of CD40 in corresponding, tumor-free specimens within nephrectomies. We also analyzed the cell surface expression and the synthesis of CD40 by cultured human ECs and studied the in vitro effects of tumor-derived angiogenic/inflammatory factors.

MATERIALS AND METHODS

RCCs and Normal Kidneys. Paired tissue specimens consisting of RCC and corresponding tumor-free kidney were collected from total nephrectomies. Specimens were frozen in isopentane and were stored in liquid nitrogen until preparation of cryostat sections or isolation of RNA. From 18 paired specimens, 6 could not be evaluated because of widespread tissue necrosis or lack of vascularized tumor tissue. In 12 nephrectomies, enough tissue was preserved suitably in both the tumor and the corresponding normal kidney to allow comparative immunohistological analysis. Renal tumors comprised 10 carcinomas of the clear-cell type and 2 oncocytomas.

A cell line derived from RCC-26 has been described in detail elsewhere (10). Cell line Caki I was established from a different RCC, and cell line Caki II was derived from a Caki I metastasis. Two cell lines derived from normal kidney have been described previously (11). The cell lines were grown in RPMI 1640 supplemented with 10% FCS, 1X nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 2 units/ml penicillin, and 2 µg/ml streptomycin (all from Biochrom, Berlin, Germany).

The cell line HeLa derived from cervical carcinoma (American Type Culture Collection CCL2) and transfected with CD40 (12) was cultured in DMEM (Life Technologies, Inc., Eggenstein, Germany), and the murine cell line A9 (13) was grown in RPMI 1640; both media contained 10% FCS (Biolog, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, and 2 µM L-α-labeled L-glutamine) (all from Life Technologies, Inc.).

For stimulation experiments, HUVECs were seeded in tissue culture flasks (Greiner, Frickenhausen, Germany) and were grown in medium containing EC growth supplement (0.4% v/v), heparin (90 µg/ml), bFGF (recombinant, human; 1 ng/ml), epidermal growth factor (recombinant, human; 0.1 ng/ml), hydrocortisone (1 µg/ml), all of the preceding from Pro-EC Culture and in Vitro Stimulation. Human umbilical cords obtained from healthy women were used for the isolation of ECs. Cryostat sections were prepared from cord specimens prior to isolation, and slices were stored at −80°C for immunohistological analysis. HUVECs were harvested by collagenase treatment according to a method by Jaffe et al. (14). Cells were seeded in tissue-culture flasks (Greiner, Frickenhausen, Germany) and were grown in medium containing EC growth supplement (0.4% v/v), heparin (90 µg/ml), bFGF (recombinant, human; 1 ng/ml), epidermal growth factor (recombinant, human; 0.1 ng/ml), hydrocortisone (1 µg/ml; all of the preceding from Promocell, Heidelberg, Germany), and FCS (10% v/v) and gentamycin (50 µg/ml; both from Biochrom).

For stimulation experiments, HUVECs were seeded in tissue flasks (Greiner) or in 24-well plates (Costar, Badhoevedorp, Netherlands) and were incubated for 3 days in the presence of or absence of cytokines or growth factors. Cells were then harvested by EDTA treatment (0.05% in PBS (1.9 mM sodium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, and 154 mM...
sodium chloride) and analyzed further by flow cytometry or Northern blot hybridization. The following recombinant human cytokines and growth factors were used: IFN-γ (1000 units/ml), TNF-α (10 ng/ml), IL-1 (100 units/ml), IL-4 (1000 units/ml), human IL-6 derived from leucocytes (1000 units/ml; all of the preceding from Boehringer Mannheim, Mannheim, Germany), bFGF (2, 10, and 20 ng/ml), VEGF (10 and 100 ng/ml; bFGF and VEGF from Biomol, Hamburg, Germany), angiogenin (1 and 100 ng/ml; Bachem, Heidelberg, Germany), erythropoietin (50 units/ml; Cilag GmbH, Sulzbach, Germany), and supernatants harvested after 24 h from RCC and normal kidney cell cultures.

In the coculture experiments, 2 × 10^6 HUVECs and 5 × 10^5 RCC cells or 2 × 10^5 cells derived from normal kidney were seeded together in 1 well of a 24-well plate (Costar) and were grown for 3 days in the EC medium before they were harvested by EDTA treatment (0.05% in PBS) and analyzed further by flow cytometry.

**Determination of TNF-α and IL-6 in Cell Culture Supernatants.** Twenty-four-h supernatants from RCC and normal kidney cell cultures were collected, immediately frozen, and stored at −80°C. In serial dilutions they were tested for TNF-α activity using a cytotoxicity assay. A9 cells, which are L cell derivatives (13), were seeded in 96-well tissue culture plates (flat bottomed; Greiner) at a density of 3 × 10^4 cells/well. Twenty-four h later, the culture medium was replaced by the supernatants and serial dilutions of them in the presence of 50 μg/ml cycloheximide (Sigma Chemical Co., Deisenhofen, Germany). After 18 h of incubation, the cells were stained using the neutral red (Sigma Chemical Co.) uptake method as described previously (15). Using serial dilutions of TNF-α as a standard, the 50% killing dose was determined (Sigma Chemical Co.).

**Microtiter plates (Nunc-Immuno MaxiSorp P96; Nunc, Wiesbaden-Biebrich, Germany) were coated at 4°C overnight with 120 μl of mouse mAbs against IL-6 [clone 34-1 (IgG1; Ref. 16); 5 μg/ml in PBS/0.02% sodium azide]. After washing with PBS/0.02% sodium azide, wells were blocked for 4 h at 37°C with 150 μl of 0.5% BSA in washing buffer containing 0.02% sodium azide. After washing, serial dilutions of cell culture supernatants and, as a standard, human IL-6 (1000 units/ml; Boehringer Mannheim) were applied. After 4 h of incubation at 37°C, followed by washing, the plates were incubated at 4°C overnight with 100 μl of affinity-purified rabbit anti-IL-6 (0.15 μg/ml in blocking buffer; Ref. 16). The plates were then washed, and peroxide-conjugated goat F(ab')2, antirabbit IgG (Dianova, Hamburg, Germany) diluted 1:2000 in washing buffer was applied. After 2 h of incubation at room temperature, followed by washing, the reaction was developed by the addition of the substrate 2,2'-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Bi- omol). Absorption was measured at a 405-nm wavelength in an ELISA reader (SLT, Lab instraments, Crailsheim, Germany).

**Antibody Preparations.** The following mouse mAbs were used in one- or two-color flow cytometric analysis and in immunohistochemistry: antihuman CD40 [clone G28-5 (IgG1); (Ref. 17)], antihuman HLA-DR [clone L2.43 (IgG2a); Becton Dickinson, Mountain View, CA], antibodies specific for human ECs [clone EN-4 (IgG1) or clone Pal-E (IgG2a); Monosan, Uden, the Netherlands], antihuman CD62E [E-selectin, endothelial leukocyte adhesion molecule 1, clone BBA1 (IgG1)], antihuman CD106 [vascular cellular adhesion molecule 1, clone B54A5 (IgG1)], both from British Biotechnology Ltd., Oxford, UK], antihuman CD54 [intercellular adhesion molecule 1, clone BA-14 (IgG2b; Ref. 18)], antihuman cytokeratin 18 [clone CK2 (IgG1); Boehringer Mannheim], antihuman CD8 [clone B811 (IgG1; Ref. 19)], and antihuman CD45 [clone T200 (IgG2b); Hybritech, Inc., San Diego, CA]. Mouse antihuman TNF-α [clone Bay × 1351 (IgG1); Bayer Diagnostics, Munich, Germany] was used as an inhibitor in stimulation experiments (1 μg/ml).

The following antisera were used as second antibodies: peroxidase-conjugated rabbit F(ab')2, antirat IgG, FITC-conjugated rabbit F(ab')2, antirabbit IgG, FITC-conjugated goat F(ab')2, antirabbit IgG (Dianova), and PE-conjugated goat F(ab')2, antimouse IgG (Dianova).

**Indirect Immunoperoxidase Staining.** An indirect immunoperoxidase staining technique was performed as described previously in detail (20). Cryostat sections (4 μm) of the tissue specimens stored at −80°C were air dried, fixed in ice-cold acetone, and washed with PBS. The sections were incubated with the first antibody and, after washing, with the peroxidase-conjugated second antibody. Finally, the specimens were stained with 3-aminon-9-ethyl-carbazole (Sigma Chemical Co.), DMSO, and H2O2 and counterstained with hematoxylin before they were embedded in glycerine gelatin (all from Merck, Darmstadt, Germany) for microscopy.

**Flow Cytometric Analysis.** For flow cytometric analysis, HUVECs were detached by EDTA treatment (0.05% in PBS) and were distributed in 96-well microtiter plates (U-form; Greiner) at 1 × 10^6 cells/well. Cells were washed in buffer solution consisting of 1% BSA and 0.01% sodium azide in PBS by repeated cold centrifugation of a 200-μl cell suspension at 250 × g for 1 min. Buffer was poured off after each centrifugation step and was refilled. HUVECs were reacted with primary antibody for 40 min at 4°C. After washing, cells were stained with the appropriate FITC-labeled second antibody (40 min at 4°C). After washing, cells were fixed in 1% paraformaldehyde in PBS and analyzed in a FACScan (Becton Dickinson) with a 488-nm Argon laser excitation wavelength and three filters for the emitted fluorescence at 530 ± 30 nm, 585 ± 42 nm, and >650 nm. A minimum of 3000 cells per sample was analyzed.

In the coculture experiments (HUVECs together with either RCC cells or normal kidney cells), HUVECs were analyzed by two-color flow cytometry. The cell mixture was likewise detached, washed, and distributed in 96-well microtiter plates (U-form; Greiner). After staining with primary antibody (mouse mAb to CD40, MHC class II, or an irrelevant antibody) and the appropriate FITC-labeled second antibody, the cell mixture was subsequently reacted with a conjugate consisting of the EC-specific mAb EN-4 and PE-labeled goat antiserum against mouse immunoglobin. The conjugate antibodies were prepared in a separate reaction applying mAb EN-4 in excess to avoid unbound PE-labeled goat antibodies that could also react with mAbs against CD40, MHC class II, or control mAb. After washing, the cells were fixed in 1% paraformaldehyde in PBS. Only cells that were stained by the conjugate (mAb EN-4 + PE-labeled second antibody) were analyzed further by setting a gate during the flow cytometry. Control experiments were performed with HUVECs or with kidney cells alone.

**Preparation of CD40 cDNA.** The vector BS SK II (+) (Stratagene, Heidelberg, Germany) containing the full-length CD40 cDNA (described in Ref. 15) was cleaved with the endonuclease EcoRI (Boehringer Mannheim) and BamHI (Life Technologies, Inc.). Cleavage products were separated on an agarose gel (Sigma Chemical Co.), and the 420-bp fragments were purified further by absorption on a DEAE-cellulose membrane. Subsequently, the cDNA was labeled following the random primer method described by Feinberg and Vogelstein (21) with 32PdATP (Amersham Corp., Braunschweig, Germany).

**Northern Blot Analysis.** Total RNA was isolated from 1 × 10^6 HUVECs stimulated or not with 1000 units/ml IFN-γ, from 1 × 10^6 cells of the cervical carcinoma cell line HeLa (CCLS; American Type Culture Collection) transfected with CD40, and from tissue specimens of RCC and the corresponding normal kidney according to the single-step method of Chomczynski and Sacchi (22). RNA was separated on a formaldehyde-containing agarose gel, blotted onto a noncharged nylon membrane (Hybond N; Amersham Corp.) and crosslinked by UV light (3 min, 302 nm). The membranes were hybridized at 65°C with the labeled 420-bp CD40 cDNA. Hybridization and washings were performed according to the method of Church and Gilbert (23).

For quantitative comparison of the total amount of blotted RNA, the membranes were hybridized with a GAPDH-specific oligonucleotide (Ref. 24). The oligonucleotide was labeled by the terminal transferase method in a single reaction containing 32PdATP (Amersham Corp.). The blots were prehybridized for 1 h at 65°C in buffer containing 7% SDS, 10× Denhardt’s solution, 20 μm sodium phosphate (pH 7.0), 5× SSC [1× SSC = 15 mm sodium citrate, 150 μm sodium chloride (pH 7.0)], and 0.2 mg/ml heat-denatured salmon sperm DNA. Hybridization was carried out in the same solution containing 100 mg/ml dextran sulfate and the labeled oligonucleotide at 65°C overnight. The blots were washed for 15 min at 65°C in a buffer containing 5× SSC, 10× Denhardt’s solution, 10 μm sodium phosphate (pH 7.0), and 3× SSC, and finally in buffer containing 1% SDS/1× SSC. Autoradiography of the hybridized blots was performed at −70°C using intensifying screens (Amersham Corp.).

**RESULTS**

**Phenotypic Characterization of ECs in RCC, in Normal Kidney Tissue, and in Umbilical Cord.** Comparable immunostaining was observed in 12 RCCs. In Fig. 1A, the rich capillary network is observed in 12 RCCs. In Fig. 1A, the rich capillary network is
Fig. 1. Strong expression of CD40 in capillaries of renal carcinoma of clear cell type. A, the rich capillary network within the tumor is revealed by immunostaining with the EC-antibody Pal-E. B, comparable staining of capillaries with mAb against CD40 (original magnification, ×150). C, nests of tumor cells are identified by the presence of cytokeratin 18. D, with rare exceptions, tumor cells are devoid of CD40, in contrast to the numerous capillaries (original magnification, ×250).
delineated by mAbs against endothelium-specific antigens. All capillaries showed strong expression of CD40 (Fig. 1, B and D). With rare exceptions, nests of tumor cells were devoid of CD40 and could be identified clearly by staining of cytokeratin 18 (Fig. 1C). Vascular expression of CD40 was not associated anatomically with the variable degree of tumor infiltration by blood mononuclear cells. Adhesion molecules CD56 and CD106 were usually present in capillaries and on tumor cells. CD62E was expressed, if ever, in single vessels within the supportive stroma (data not shown).

In normal kidney tissue, although rich in interstitial capillaries (Fig. 2A), CD40 was not seen on ECs but was seen regularly in certain tubular segments and in few interstitial cells (Fig. 2B). Some of these cells may also belong to peritubular capillaries. Glomerula did not express CD40. Endothelia within normal kidney expressed CD54, but not CD106 or CD62E, as described in previous studies (25, 26).

Endothelia within 12 umbilical cords showed a heterogeneous staining pattern, in that CD40 was absent in five specimens and produced very faint staining in seven samples. There was also heterogeneity with respect to the distribution of adhesion molecules. The majority of vessels were positive with CD54 and less with CD62E, whereas CD106 was largely absent. Immunohistological results are summarized in Table 1.

**Induction of Endothelial CD40 in Vitro.** Expression and inducibility of endothelial CD40 in vitro were studied with HUVECs. Isolated HUVECs in culture showed a low, basal cell surface expression of CD40 that was increasing slightly during cell culture.

### Table 1 Immunostaining of ECs in paired tissue samples from RCC and normal kidney (n = 12) and umbilical cords (n = 12)

<table>
<thead>
<tr>
<th></th>
<th>Capillaries in RCC</th>
<th>Capillaries in normal kidney</th>
<th>Endothelium in umbilical cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pal-E</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD40</td>
<td>+</td>
<td>+</td>
<td>(+ )</td>
</tr>
<tr>
<td>MHC class II</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CD54</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD106</td>
<td>(+ )</td>
<td>-</td>
<td>(+ )</td>
</tr>
<tr>
<td>CD62E</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*Pal-E, EC-specific mAb; +, positive, ubiquitous staining; (+), positive staining restricted to certain segments; −, absent staining.*
Table 2 Differential induction of CD40 and MHC class II products on cultured HUVECs by various cytokines/angiogenic molecules (flow cytometric analysis, three separate experiments)

<table>
<thead>
<tr>
<th>Expression on HUVECs</th>
<th>CD40</th>
<th>MHC class II</th>
<th>CD54</th>
<th>CD62E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated HUVECs</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IL-6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>bFGF</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>VEGF</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Supernatant RCC-26 cells</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant normal kidney cells</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Coculture with RCC-26 cells</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Coculture with normal kidney cells</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*+, enhanced expression; -, negative or unchanged expression; +/-, variable, low expression; ND, not determined.

(data not shown). This expression of CD40 could be further upregulated with IFN-γ, TNF-α, IL-1, and IL-6, but not with IL-4 and the angiogenic growth factors bFGF (27), VEGF (28), erythropoietin (29), and angiogenin (Ref. 30; Table 2; Fig. 3). Induction of endothelial CD40 by TNF-α could be inhibited by a mAb against TNF-α. HUVECs did not express MHC-class II molecules during cell culture; this expression was only stimulated with IFN-γ but not with the other agents listed in Table 2. Expression of CD54 increased after stimulation with IL-1, TNF-α, and IFN-γ, whereas IL-4 and IL-6 did not alter the CD54 expression. CD62E expression increased after stimulation with IL-1 or TNF-α. IL-4, IL-6, and IFN-γ had no effect on CD62E expression (data not shown).

To study the effects of carcinoma-derived factors, HUVECs were incubated with supernatants from cultures of RCC and normal kidney cells. Only supernatant from the RCC-26 cell line induced the expression of CD40 on HUVECs (Fig. 4), whereas supernatants from cell lines Caki I and Caki II and from the normal kidney cell lines had no effect (data not shown). Similar to TNF-α, IL-1, and IL-6, supernatant...
from RCC-26 did not concomitantly induce the expression of endothelial MHC class II molecules. Addition of mAbs to TNF-α did not, however, inhibit the induction of endothelial CD40 by RCC-26 supernatant in three experiments (data not shown). Supernatants from RCC-26 and normal kidney cells enhanced the expression of CD54 but did not alter the expression of CD62E on HUVECs.

In an attempt to provide a continuous exposure to stimulating factors, thus mimicking the situation in vivo, HUVECs were also cocultured for 3 days together with RCC cell lines and normal kidney cells. In these experiments, a clear distinction between HUVECs and cocultured RCC-26 cells resulted in up-regulation of CD40 on HUVECs (Fig. 4). Supernatants from RCC-derived RNA hybridized clearly with the CD40 cDNA, when 20—30 μg RNA were applied (data not shown). The RCC-derived RNA hybridized clearly with the CD40 cDNA, when 20—30 μg of RNA were applied (Fig. 7).

**DISCUSSION**

This study demonstrates a striking difference in the phenotype of capillaries within RCC and of capillaries within the corresponding, tumor-free renal tissue, in that all tumor-associated endothelia strongly expressed CD40, whereas blood vessels in normal kidney sections were devoid of CD40. That endothelial CD40 is in fact related to the presence of tumor cells could be substantiated by in vitro experiments, when a cell line derived from RCC and its supernatant, but not normal kidney cells, could induce CD40 on ECs in culture.

RCCs are malignant tumors that show extensive vascularization and capillary proliferation. It is not known at the moment which of the tumor-derived angiogenic factors identified so far (31) are mainly responsible for the neovascularization in RCC. Apart from angiogenic factors, RCCs also produce a variety of pleiotropic cytokines, preferentially IL-6 and TNF-α (32), which may cause the “inflammatory” clinical presentation, including fever and the elaboration of acute-phase reactants. Among them, especially TNF-α is endowed with angiogenic activity in tumors (33). Thus, the expression of capillary CD40 theoretically could be associated with inflammation, with angiogenesis, or with both qualities. Two recent studies (8, 9) have attributed endothelial CD40 to vascular inflammation, and in one study (9), the enhanced expression of vascular CD40 within inflammatory skin lesions and within Kaposi’s sarcoma (34) was demonstrated. The results presented in this study suggest, however, that within RCC, the appearance of endothelial CD40 may also be related to angiogenesis. First, the widespread and even distribution of CD40 in tumor capillaries, showing no apparent anatomical association with infiltrating blood mononuclear cells, argues strongly against vascular inflammation. Inflammatory endothelial changes are usually distrib-
Fig. 5. Induction of endothelial CD40 during coculture with RCC-26 cells. Two-color flow cytometric analysis of HUVECs after 72 h of coculture. Identification of HUVECs within the cell mixture by conjugate antibodies (mAb EN-4 + PE-goat anti-sera; FL2). A, negative staining of RCC-26 cells by conjugate antibodies; B, clear staining of HUVECs by conjugate antibodies allows for separate analysis by setting an appropriate gate. C, low constitutive expression of CD40 on HUVECs cultured in medium; D, up-regulation of endothelial CD40 during coculture with RCC-26 cells. Solid lines, staining with CD40 mAb; dotted lines, staining with irrelevant mAb as isotype control (FL1).

Table 3 Determination of IL-6 in 24-h supernatants of RCC and normal kidney cell culture

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Concentration of IL-6 (units/ml)</th>
</tr>
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<tbody>
<tr>
<td>Normal kidney (NN21)</td>
<td>50 ± 10(^\text{a})</td>
</tr>
<tr>
<td>RCC-26</td>
<td>50 ± 11</td>
</tr>
<tr>
<td>Caki I</td>
<td>270 ± 50</td>
</tr>
<tr>
<td>Caki II</td>
<td>70 ± 20</td>
</tr>
</tbody>
</table>

\(^{a}\) Mean values of two experiments.

Fig. 6. Detection of CD40-specific RNA in HUVECs by Northern blot analysis. Only RNA derived from HUVECs stimulated with IFN-\(\gamma\) yields a hybridization signal with CD40 cDNA. RNA from unstimulated HUVECs does not hybridize with CD40 cDNA. Control hybridizations with GAPDH-specific oligonucleotides indicate that roughly equal amounts of RNA have been applied.

Fig. 7. Detection of CD40-specific RNA in tissue specimens from RCC by Northern blot analysis. There is absent hybridization of CD40-specific cDNA probe with total RNA prepared from normal kidney tissue (10 and 20 \(\mu\)g applied) and a distinct hybridization signal with RNA from corresponding RCC (10, 20, and 30 \(\mu\)g applied). Control hybridizations were performed with GAPDH-specific oligonucleotides.
potentially produced in RCC (32) can upregulate endothelial CD40 in vitro (8, 9). However, according to our in vitro experiments, the action of IFN-γ, TNF-α, IL-1, and IL-6 in this setting is highly unlikely. IFN-γ concomitantly induced endothelial MHC class II products, whereas the factor(s) derived from RCC-26 did not upregulate endothelial MHC class II. An independent expression of CD40 and MHC class II products has previously been noticed also with carcinomas and B-cell lines (35). TNF-α could clearly be excluded, because it was not contained within cell supernatants as determined by a sensitive cytotoxicity assay, and induction of CD40 could not be inhibited by mAbs against TNF-α. IL-1, in contrast to the factor(s) derived from RCC-26, also upregulates the expression of CD62E. Finally, IL-6 could not account for the selective effects observed with RCC-26 as it was detectable in the supernatants from all three RCC cell lines and in concentrations far below that required for induction of CD40. Studies are in progress to further determine a single factor in RCC that could be responsible for the induction of endothelial CD40, but possibly different factors operate in different tumors in vivo.

Notwithstanding the principal induction pathway of endothelial CD40, the prominent expression in capillaries of RCC is intriguing. It is well known that RCCs are often infiltrated by lymphocytes that are believed to combat the tumor. Therefore, augmentation of cellular immunity in patients is a major goal of current therapeutic strategies using cytokines or genetically modified tumor cells (36). It is tempting to speculate that endothelial CD40 could engage with CD40 ligand on immune cells that are present within the tumor. The enhanced expression of adhesion molecules, thereby promoting leucocyte extravasation and tumor infiltration, could be one consequence of endothelial activation via CD40. Whether additional, as yet undefined endothelial functions would operate after ligation of CD40 remains to be determined.

As defined by cytofluorimetric analysis, umbilical vein endothelial cells in culture showed a constitutive, low cell surface expression of CD40. Because cells were grown in culture medium containing several growth factors before experiments were performed, the observed basal expression might result from in vitro stimulation. ECs do not appear to express CD40 in vivo under physiological conditions, as could be shown by negative or very faint immunostaining in tissue sections from corresponding umbilical cords or from normal kidney. However, the stimulatory effect of several cytokines in vitro suggests that potentially all ECs have the capacity to produce and carry CD40.

The presence of CD40 in certain tubular segments and in few interstitial cells within normal kidney tissue warrants additional studies. Although epithelial cells can express CD40 (7), the observed tubular staining could as well represent reabsorption of soluble, urinary CD40. The Northern hybridization results, showing CD40-specific RNA in tumor tissue only, but not in normal kidney, are compatible with an uptake of CD40 by renal tubules. However, an active production in normal kidneys is not excluded entirely by this method, because the level of specific transcription may be below detection threshold, and more refined PCR techniques have not been performed up to now.

In conclusion, CD40 has been identified as an EC surface molecule that is expressed strongly in the tumor-associated capillaries within RCCs. In this setting, the histological presentation in vivo and the mode of induction in vitro attribute endothelial CD40 to angiogenesis rather than to inflammation. As a central immunoregulatory molecule, CD40 could serve as a link between the vascular endothelium and the immune system in malignant diseases.

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