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Proangiogenic Function of CD40 Ligand-CD40 Interactions¹,²

Marlies E. J. Reinders, Masayuki Sho, Stuart W. Robertson, Christopher S. Geehan, and David M. Briscoe

Angiogenesis is a characteristic component of cell-mediated immune inflammation. However, little is known of the immunologic mediators of angiogenesis factor production. Interactions between CD40 ligand (CD40L) and CD40 have been shown to have pluripotent functions in inflammation, including the production of cytokines, chemokines, as well as the angiogenesis factor, vascular endothelial growth factor (VEGF), by endothelial cells. In this study we found that treatment of cultured human endothelial cells with an anti-CD40 Ab (to ligate CD40) resulted in the expression of several other angiogenesis factors, including fibroblast growth factor-2 and the receptors Flt-1 and Flt-4. To determine the proangiogenic effect of CD40L in vivo, human skin was allowed to engraft on SCID mice for 6 wk. These healed human skins express CD40 on resident endothelial cells and monocyte/macrophages, but not on CD20-expressing B cells. Skins were injected with saline, untransfected murine fibroblasts, or murine fibroblasts stably transfected with human CD40L. We found that the injection of CD40L-expressing cells, but not control cells, resulted in the in vivo expression of several angiogenesis factors (including VEGF and fibroblast growth factor) and a marked angiogenesis reaction. Mice treated with anti-VEGF failed to elicit an angiogenesis reaction in response to injection of CD40L-expressing cells, suggesting that the proangiogenic effect of CD40L in vivo is VEGF dependent. These observations imply that ligation of CD40 at a peripheral inflammatory site is of pathophysiological importance as a mediator of both angiogenesis and inflammation. The Journal of Immunology, 2003, 171: 1534–1541.

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Abbreviations used in this paper: EC, endothelial cell; CD40L, CD40 ligand; FGF, fibroblast growth factor; PCNA, proliferating cell nuclear Ag.; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.


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In addition, some of the intracellular signaling pathways for CD40-dependent angiogenesis have recently been defined (35, 37). Together, these in vitro studies point to a major function for CD40L-CD40 interactions in angiogenesis.

In this study we extended upon these observations in an in vivo model and tested the mechanism of function of CD40L for angiogenesis in vivo. Our findings clearly demonstrate that the provision of CD40L to tissues in vivo is a physiologic stimulus for the production of several angiogenesis factors, but that the CD40L-induced angiogenesis reaction is VEGF dependent.

Materials and Methods

Reagents

The following Abs were used in these studies: anti-human CD31, anti-von Willebrand factor (anti-vWf), anti-CD20, anti-CD68, and anti-mouse CD31 (DAKO, Carpenteria, CA); anti-human CD40 (G28.5) and CD40L (106; gifts from D. Hollenbaugh, Bristol Myers Squibb, Princeton, NJ); and anti-human CD40L, VEGF, and FGF-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Humanized anti-human VEGF for in vivo studies was a gift from Genentech (South San Francisco, CA). Human IgG was purchased from Sigma-Aldrich (St. Louis, MO). The proliferating cell nuclear Ag (PCNA) kit was purchased from Novocastra (Burlingame, CA). Murine fibroblasts stably transfected with human CD40L (CD40L cells) or untransfected cells (mock cells) were a gift from C. van Kooten (38).

Cell culture

Single-donor human umbilical vein EC were purchased from Clonetics (Walkersville, MD) and were cultured in complete endothelial medium (EGM Bulletkit; Clonetics) as supplied and according to the recommended instructions. EC were subcultured and used at passages four to six.

In vivo assessment of angiogenesis

CB.17 SCID mice were purchased from Taconic Farms (Germantown, NY) and were used at 6–8 wk of age. The SCID mice used in these studies were occasionally monitored for "leakiness" by assessment of circulating T cells, and in all cases were found to be immunodeficient and stable. Full-thickness human neonatal foreskin grafts were transplanted onto CB.17 (SCID) mice as previously described (25, 39). Following engraftment for

FIGURE 1. CD40 ligation induces FGF-2, Flt-4, and Flt-1 in EC in a time- and dose-dependent manner. Confluent cultures of human EC were treated for 6 h with different concentrations of stimulating anti-CD40 (A, C, and D) or as a time course using 1 μg/ml anti-CD40 (B) as indicated. Total RNA was harvested, and the expression of angiogenesis factors was analyzed by RNase protection assay and PCR. Anti-CD40 induced the expression of FGF-2, Flt-4, and Flt-1 expression in EC (A and B), whereas several other angiogenesis factors and receptors had a constitutive level of expression and no detectable change after treatment with anti-CD40 (C and D). mRNA expression was quantified by densitometry as the relative expression of the angiogenesis factor compared with the expression of the housekeeping GAPDH signal. Bar graphs on the right of A and B illustrate the relative expression of each gene examined (mean ± 1 SD of three blots, including the illustrated blots). The expression of GAPDH or β-actin served as internal housekeeping gene controls. All autoradiographs are representative of three experiments with similar results.
4–6 wk, mice were divided into different experimental groups. In each group the human skin was injected intracutaneously with 35 μl of Matrigel (growth factor-reduced Matrigel; BD Biosciences, Bedford, MA) and either 35 μl of saline (as a control) or 2.5 × 10⁶ murine fibroblasts stably transfected with human CD40L (CD40L cells) or mock transfectants (mock cells) each in 35 μl of saline. Before injection, mock and CD40L cells were irradiated at 7500 rad.

SCID mice were also treated with a neutralizing anti-human VEGF Ab (Genentech) or human IgG at a dose of 5 mg/kg in 100 μl of saline i.p. Ab therapy was begun 2 days before and every other day after the injection of CD40L cells into the skin. Animals were sacrificed, and skin grafts were harvested after 7 days. Animal care and anesthesia were performed in compliance with guidelines established by the animal care and use committee at Children’s Hospital (Boston, MA).

**Immunohistochemistry**

Frozen specimens were fixed in acetone, and formalin-fixed specimens were deparaffined. All specimens were then incubated in primary and secondary Abs as previously described (25, 33) using the Vectastain Kit (Vector Laboratories, Burlingame, CA). Finally, specimens were counterstained in Gill’s hematoxylin and mounted in glycerol gelatin. The PCNA kit was used according to the manufacturer’s instructions.

**Quantification of angiogenesis**

Vessels were identified by staining for human vWF, and angiogenesis was quantified by a standard grid-counting method at ×400 magnification as previously described (40). Four to six adjacent nonoverlapping fields of each specimen were analyzed in a blinded manner, and the mean vessel counts for each specimen were calculated.

**RT-PCR and RNase protection assays**

RNA was isolated from cultured EC or skin samples using the Ultraspec RNA isolation system (Biotex, Houston, TX) and was reverse transcribed, and PCR was performed using standard techniques (33). Sequence-specific primers for PCR of human KDR (also called VEGF receptor-2) were: sense, 5’-CGAAGCATACATGAAAGAAAC-3’; and antisense, 5’-ACATACACACACAGGAG-3’. β-Actin (Stratagene, La Jolla, CA) was used as an internal control. The PCR conditions were 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The last cycle was extended to 7 min at 72°C. The amplified products were resolved by electrophoresis in an ethidium bromide-stained 1.5% agarose gel.

RNase protection assays were performed using the RibOQuant multiprobe template system (BD PharMingen, San Diego, CA), according to the manufacturer’s instructions and as previously described (33). Briefly, equal amounts of RNA (10 μg) were hybridized with [32P]UTP-labeled riboprobes that were synthesized from the template. After hybridization, samples were digested with RNase A, RNase T1, and proteinase K, and protected RNA was resolved on a denaturing polyacrylamide gel. Relative signals were detected by autoradiography with Kodak MR film (Eastman Kodak, Rochester, NY), and expression was quantified by densitometry by means of an Alphalager 2000 system (Alpha Innotech, San Leandro, CA). For quantification, signals were standardized to the internal housekeeping gene GAPDH.

**Statistical analyses**

Data were compared by nonparametric analysis using the Mann-Whitney test for data analysis. Differences with p < 0.05 were considered statistically significant.
Results

Ligation of CD40 induces FGF-2, Flt-4, and Flt-1 mRNA expression in human EC in vitro

We first assessed the effect of CD40L-CD40 interactions on the expression of a panel of angiogenesis factors and receptors in cultured EC in vitro using RNase protection assays. Human EC were starved overnight in 0.5% FCS and were subsequently treated with stimulating anti-CD40. Anti-CD40 induced the expression of FGF-2, Flt-1 and Flt-4 in a dose- and a time-dependent manner (Fig. 1, A and B). FGF-2, Flt-1, and Flt-4 increased in expression as early as 3 h following activation with anti-CD40, with peak expression occurring after 6 h (Fig. 1B). Similar results were obtained following treatment with soluble CD40L, and under all conditions resulted in the expression of VEGF (data not shown) (33). In contrast, the mRNA expression of angiopoietin, endoglin, CD31, TIE, TIE2, thrombin receptor, and KDR was unaltered after treatment with anti-CD40 (Fig. 1, C and D). These findings suggest that CD40L-CD40 interactions have the potential to be potent for angiogenesis in vivo.

Infiltration of tissues with CD40L-expressing cells induces angiogenesis in vivo

We next assessed whether cell surface CD40L on infiltrating cells might function to mediate immune-mediated angiogenesis in vivo. It is well established that activated platelets and lymphocytes expressing CD40L are present in tissues in cell-mediated immune inflammation. We used an established model of angiogenesis in which human skin is transplanted onto SCID mice (25). Skin is allowed to heal for 4–6 wk to enable stabilization of the healing angiogenesis reaction in the skin graft (39). Untransfected murine fibroblasts (mock cells) or murine fibroblasts stably transfected with human CD40 ligand (CD40L cells; Fig. 2, A and B) were injected intracutaneously into the healed human skins on the SCID mice. Injection of saline served as a negative control. The skins were harvested after 7 days and were evaluated for the development of angiogenesis. By quantitative grid counting, we found that the number of vessels in skins injected with saline or mock cells was comparable to that in untreated skins, as determined by standard immunostaining with vWF. In contrast, the mean vessel density was significantly increased in the skins injected with CD40L cells vs controls (2.3-fold vs saline and 2.1-fold vs mock cells, both p < 0.01; Fig. 2, C–E). Similar to untreated human skins (39), the skins injected with mock-transfectant cells had an approximately equal number of human and mouse EC, as determined by immunohistochemical analysis of human vs mouse CD31. In contrast, we found a greater percentage of cells expressing human CD31 in skins treated with CD40L cells (not shown). Furthermore, in the CD40L-injected skins there were numerous EC expressing PCNA in their nucleus, a marker that identifies cells that have entered the cell cycle (Fig. 2, F and G). This suggests active angiogenesis in skins that received CD40L cells.

We also wished to establish which intragraft cells may respond to the injected CD40L cells. By immunohistochemistry, CD40 was expressed by multiple cells in untreated skins, including EC, resident macrophages, and keratinocytes with a similar pattern of expression in all groups of skins (Fig. 3). CD68-expressing monocyte/macrophages were present in all skins examined. There was no staining of CD20 in any of the skin samples (middle panel, left), whereas CD68-expressing monocyte/macrophages (lower panels) were present in all skins examined. The expression of CD68 was similar in mock- and CD40L-treated skins. Data are representative of three skins examined in each group. Magnification of micrographs: ×200 (upper left panel) and ×400.

FIGURE 3. Expression pattern of CD40-expressing cells within skin grafts: representative immunohistochemical staining of CD40, CD40L, CD20, and CD68 in untreated skins (Untreated) or skins treated with CD40L-transfected cells (Treated). CD40 (upper panels) was expressed on EC and resident macrophages in all skins examined. There was no difference in the pattern or intensity of expression of CD40 in untreated or treated skins. In contrast, CD40L was present only in the skins treated with the CD40L transfectants, where its expression was most apparent on groups of cells in clusters (middle panel, right). There was no staining of CD20 in any of the skin samples (middle panel, left), whereas CD68-expressing monocyte/macrophages (lower panels) were present in all skins examined. The expression of CD68 was similar in mock- and CD40L-treated skins. Data are representative of three skins examined in each group. Magnification of micrographs: ×200 (upper left panel) and ×400.
in untreated skins and in skins injected with mock transfectant cells. In contrast, staining for CD40L was found on discrete clusters of cells in the skins injected with CD40L transfectants (Fig. 3). By double immunohistochemical staining (not shown), there was no distinct pattern of contact between the CD40L-expressing clusters and EC or macrophages. We interpret this to suggest that the injected CD40L-expressing cells may interact with several resident CD40-expressing skin cells spatially associated with the CD40L cell clusters. Alternatively, it is possible that CD40L shed from these cells may bind the several CD40-expressing cells within the skin to mediate the angiogenesis reaction.

**CD40L induces the expression of multiple angiogenesis factors in vivo**

We next examined the mRNA expression of a panel of angiogenesis factors and receptors (including VEGF, FGF, and angiopoietin-1) in skins harvested 7 days after intracutaneous injection of saline, mock cells, or CD40L cells. There was a low and variable expression of all factors in skins injected with saline or mock cells (Fig. 4, A–C). In contrast, the angiogenesis factors VEGF, FGF, and angiopoietin-1 were significantly up-regulated after treatment with CD40L cells. Also, the angiogenesis receptors TIE-2, Flt-4, and Flt-1 as well as the EC markers endoglin and CD31 were significantly increased in skins injected with CD40L cells (Fig. 4, A–C).

By immunohistochemistry, little FGF-2 and VEGF was found in saline- and mock cell-treated skins (Fig. 5, A–D). In contrast, a marked induction of both FGF-2 and VEGF expression was found in skins treated with CD40L cells (Fig. 5, E and F). FGF-2 and VEGF were expressed mainly by vascular EC. Thus, it is likely that CD40L-induced angiogenesis in vivo involves the expression and function of multiple angiogenesis factors.

**Function of VEGF in CD40L-induced angiogenesis**

It is well established that angiogenesis factors mediate angiogenesis synergistically with VEGF (41). Thus, it is possible that VEGF is a critical factor in CD40L-induced angiogenesis. To test this possibility, human skins on SCID mice were injected with either saline or CD40L cells, as described above. Simultaneously, the mice were treated with either anti-human VEGF or IgG as a control by i.p. injection. There was no change in baseline vessel density in anti-VEGF or IgG-treated mice when skins were treated with mock cells (not shown). In contrast, skin treated with CD40L cells in IgG-treated mice had significantly increased vessel numbers over baseline saline-treated skins (Fig. 6; p < 0.01). However, injection of CD40L cells into skins on mice treated with anti-VEGF failed to result in an angiogenesis reaction (Fig. 6; p < 0.03). Thus, the effect of CD40L for the stimulation of an angiogenesis reaction involves the critical expression of VEGF.

**Discussion**

In this study we show that local infiltration of skin with cells expressing human CD40L on their cell surface mediates the induction of VEGF and FGF in vivo and results in a marked angiogenesis reaction. These findings have important implications for the function of CD40L-CD40 interactions in immunity and chronic inflammation. First, it is well established that CD40L-CD40 interactions are of central importance in the development of an effective immune response involving T cell activation, T cell-B cell interactions, and Ab switching as well as endothelial activation responses (30–32). Our findings here extend the functions of CD40L-CD40 to include regulation of VEGF and FGF as well as the angiogenesis receptors Flt-1 and Flt-4 in vitro and in vivo. Second, inflammatory cells, including lymphocytes and monocytes, can elicit an angiogenesis reaction, a process called leukocyte-induced angiogenesis (24, 25). This process is well characterized in the literature and is reported to be of pathological significance in several chronic inflammatory diseases, including arthritis, atherosclerosis, and diabetes (1, 3, 42–44). Our finding here that CD40L-expressing cells within a tissue can functionally induce VEGF-dependent angiogenesis is consistent with these reports and further suggests that CD40L-CD40 interactions may represent an intermediary between cell-mediated immune inflammation and angiogenesis.

Activated T cells and platelets express CD40L, and CD40 is expressed on multiple cell types, including EC, fibroblasts, monocytes, and epithelial cells (45, 46). Our findings demonstrate that CD40L promotes the functional expression of several angiogenesis factors.
factors in vivo. These findings are consistent with in vitro observations by several groups that CD40L-CD40 interactions may result in angiogenesis via enhanced VEGF, tissue factor, or matrix metalloproteinase expression and via signaling through the phosphoinositide 3-kinase/Akt pathway (33–37, 47). However, despite these observations, a recent report by Urbich et al. (48) suggested that CD40L may have antiangiogenesis effects. In the report the authors cited that a possible reason for their different findings was the cell culture conditions used in their analysis. Nevertheless, our studies here demonstrate that cell surface CD40L is sufficient to facilitate angiogenesis factor expression and angiogenesis in vivo. Thus, we suggest that it is most likely that the physiological effect of CD40L-CD40 interactions is proangiogenic. However, in light of the report by Urbich (48), it will be important to determine whether there is a factor or physiologic condition that can limit or inhibit this proangiogenic effect of CD40L.

The expression of CD40 has been reported to be prominent in processes known to be associated with angiogenesis and inflammation (32, 45, 46). These include atherosclerosis, arthritis, as well as allografts undergoing rejection (49, 50). Interestingly, these same disease processes have been found to be associated with intense VEGF expression (43, 44). Moreover, FGF has been shown to act directly on smooth muscle cells, which might have important implications for inflammatory disorders, such as atherosclerosis and graft arteriosclerosis (51). Furthermore, blockade of CD40L-CD40 interactions has been found to prevent the development of acute and chronic inflammation, including allograft rejection and atherosclerosis (52–56). It is thus possible that these effects may in part be related to the CD40L-dependent angiogenesis response. Therefore, our findings here that local infiltration of CD40L-expressing cells at a peripheral inflammatory site mediate both angiogenesis factor production and angiogenesis are probably of pathophysiological importance.

In conclusion, in this report we define a role for CD40L-CD40 interactions in angiogenesis in vitro and in vivo, and we suggest that a major component of immune-mediated angiogenesis is VEGF dependent. Our findings imply that the infiltration of cells
CD40-DEPENDENT ANGIOGENESIS

References


