Inhibition of Hepatic Endothelial E-Selectin Expression by C-raf Antisense Oligonucleotides Blocks Colorectal Carcinoma Liver Metastasis

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Abstract

Cytokine-dependent induction of E-selectin expression is mediated through cooperative signaling involving the Ras/Raf/mitogen-activated protein kinase pathway. We previously reported that metastatic tumor cells entering the hepatic circulation rapidly induce a cytokine cascade leading to E-selectin induction (A-M. Khatib, et al., Cancer Res., 59: 1356–1361, 1999). Here, we investigated the effect of a blockade of E-selectin induction on colorectal carcinoma metastasis using rodent (host)-specific C-raf antisense oligonucleotides and human colorectal carcinoma CX-1 cells. Pretreatment of hepatic endothelial cells in vitro with the antisense oligonucleotides abrogated E-selectin-dependent CX-1 adhesion. In vivo, pretreatment of nude mice with these oligonucleotides abrogated E-selectin induction in response to intrasplenic/portal inoculation of CX-1 cells, and this reduced the number of liver metastases by 86% relative to controls. The results suggest that the inhibition of tumor-induced, hepatic microvessel E-selectin expression may provide a useful strategy for the prevention of hepatic metastasis.

Introduction

The liver is a major site of metastases for some of the most common human malignancies, carcinomas of the gastrointestinal tract and colorectal carcinoma in particular. Liver metastases are frequently inoperable and are associated with poor prognosis (1). The metastatic cascade involves a sequence of steps including invasion of local host tissues, entry into the circulation, arrest and adherence in the vascular bed, and extravasation into the target organ parenchyma (2). The evidence suggests that the attachment of circulating tumor cells to the vascular endothelium of the target organ may be a key event in regulating extravasation (3); implicated in this adhesion are site-specific microvascular endothelial cell-surface molecules (4) and cytokine-inducible receptors that are normally involved in inflammation-induced leukocyte adhesion and transmigration (5). Among the cytokine-inducible receptors implicated in leukocyte transmigration and tumor metastasis are the selectins, E-selectin in particular (4). The expression of this molecule on vascular endothelial cells is induced by proinflammatory cytokines such as IL-1β (1) and TNFα (6). These cells express type 1 (TNFR60) and type 2 (TNFR80) TNF receptors, but the former is thought to be the major form involved in soluble TNFα-induced cellular responses (7, 8). Signaling through this receptor appears to involve activation of the p42 ERK, p38 MAPK, and p54 JNK pathways (8, 9) as well as NFκB activation (10) and may depend on cooperative signaling between these pathways. Recent studies have implicated the Ras and Raf kinases that act upstream of the MAPK pathway in transcriptional activation of E-selectin, an activity that may be secondary to a TNFα-induced increase in ceramide production (8, 9).

Previously, we have shown that highly metastatic cells entering the liver can rapidly induce a cytokine cascade involving activation of TNFα production, and this leads to up-regulation of hepatic sinusoidal endothelial E-selectin expression and subsequently of ICAM-1 and VCAM-1 (11). Using an E-selectin-specific monoclonal antibody, we have also shown that E-selectin is involved in metastases formation in this organ (12). Here, we asked whether a blockade of tumor-induced endothelial E-selectin expression could inhibit liver metastases formation. Previously, it was shown that suppression of C-raf kinase in microvascular endothelial cells using an ASO approach was effective in preventing cytokine-mediated up-regulation of cell adhesion molecules, including E-selectin, and the efficacy of this approach in vivo was demonstrated in a rat heart allograft survival model (13, 14). To block E-selectin expression in vivo, we, therefore, used rodent-specific C-raf kinase ASO. We show here that the ASO blocked adhesion of human colorectal carcinoma CX-1 cells to murine hepatic endothelial cells and inhibited experimental liver metastasis.

Materials and Methods

Mice. Unless otherwise indicated, 6–8-week-old female NIH Swiss nu/nu mice (Taconic Labs, Germantown, NY) were used for all of the experiments. The mice were housed in a pathogen-free facility, fed with sterilized food and water, and maintained in accordance with McGill University Animal Care guidelines. For some of the experiments, 6–8-week-old female C57Bl/6 mice from Charles River Laboratories (Wilmington, MA) were used.

Cell Lines. Human colorectal carcinoma lines CX-1 (highly metastatic) and MIP-101 (poorly metastatic) were developed from two colon carcinoma biopsies (15) and were a kind gift from Dr. Peter Thomas (Boston University School of Medicine, Boston, MA). The cells were maintained in RPMI medium containing 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml of glutamine. Routine testing confirmed that the cells were free of Mycoplasma and viral contaminants during the study period.

Reagents. Rat and mouse C-raf antisense (C-raf ASO; ISIS 15770-10) and a combinatorial mixture of oligonucleotide sequences made of the same chemistry as ISIS 15770 (ISIS 29848-5, control oligo; Ref. 14) were provided by ISIS Pharmaceuticals (Carlsbad, CA). They were prepared in saline at the desired concentration, before use in the in vitro and in vivo experiments. Recombinant human TNFα was from R&D Systems (Minneapolis, MN). Induction of murine hepatic endothelial cell E-selectin expression by this cytokine was documented previously (11). Na235Cr came from Perkins Elmer Life Sciences (Boston, MA).

MTT Assay. Cell proliferation was measured by the MTT assay as was described previously (16). Cells were seeded in 24-well plates at a density of 5 × 103 cells/well and cultured overnight in RPMI containing 10% serum before the addition of the oligonucleotides and incubation for up to 3 days.
we described previously (12). Adhesion assays were performed using Na\(^{32}\)Cr
labeled tumor and TNFα-treated endothelial cells (12). To test the effect of C-raf ASOs on tumor-endothelial cell adhesion, the endothelial cells were cultured for 5 days, and the culture medium was removed and replaced with Opti-Mem medium containing 3 µl of LipofectAMINE (both from Life Technologies, Inc., Burlington, Ont.) with or without different concentrations of the oligonucleotides and incubated for 5 h at 37°C. The medium was aspirated and was replaced with RPMI (10% FCS), and the cells were incubated for 48 h before the adhesion assay.

**RNA Extraction, Northern Blot Analysis, and RT-PCR.** RNA extraction from liver specimens, RT-PCR, and the cytokine and E-selectin primers were described in detail elsewhere (11). To determine the effect of oligonucleotide treatment on tumor-induced E-selectin expression, nude or C57Bl/6 mice received injections i.v. of 25 mg/kg oligonucleotides 24 and 4 h before tumor cell treatment on tumor-induced E-selectin expression, nude or C57Bl/6 were described in detail elsewhere (11). To determine the effect of oligonucleotides and incubated for 5 h at 37°C, the medium was replaced with RPMI (10% FCS), and the cells were incubated for 48 h before the adhesion assay.

The mice were inoculated with 2 × 10⁶ CX-1 cells and splenectomized 1 min later. They were killed 4–6 weeks later, and liver metastases were enumerated immediately, without prior fixation. The oligonucleotide treatment consisted of two injections of 25 and 6 mg/kg antisense (or control) oligonucleotides 24 h and 4 h, respectively, before tumor cells injection, one injection of 6 mg/kg oligonucleotides 4 h later, and weekly injections of 25 mg/kg oligonucleotides from day 3 onward until the end of the experiment. An additional control group was given injections with vehicle (saline) only. All of the ASO and saline injections were administered i.v. (tail vein).

Results

**CX-1 Cell Adhesion to TNFα-activated Hepatic Sinusoidal Endothelial Cells Is Blocked by Pretreatment with Murine C-raf ASOs.** Previously, we have shown that CX-1 cells can adhere to TNFα-activated murine hepatic sinusoidal endothelial cells in an E-selectin-dependent manner (11). Because C-raf was implicated in the regulation of TNFα-dependent E-selectin induction, we investigated whether pretreatment of the endothelial cells with C-raf ASO can inhibit TNFα–dependent E-selectin expression and CX-1 adhesion. Hepatic endothelial cells were treated with different concentrations of C-raf ASO for 4 h, cultured for an additional 48 h, and then stimulate with 50 ng/ml TNFα for 5 additional hr to induce E-selectin. In these cells, C-raf and E-selectin expression as determined

[Fig. 1. C-raf ASOs block CX-1 cell adhesion to TNFα-activated hepatic sinusoidal endothelial cell. Primary murine hepatic endothelial cells cultured in 24-well plates were incubated for 5 h at 37°C with oligonucleotides in Opti-Mem containing 3 µl of LipofectAMINE. The medium was replaced with RPMI containing 10% FCS for an additional 48 h incubation at 37°C, and 50 ng/ml TNFα were added to induce E-selectin. RNA was extracted for RT-PCR analysis (top) 2 h, and adhesion of Na\(^{32}\)Cr-labeled CX-1 cells (bottom) was measured 5 h after the addition of TNFα. The bar graphs, results of laser densitometry performed on the cDNA bands. They are expressed as C-raf/GAPDH (A) and E-selectin/GAPDH (B) density ratios. The proportion of adhering cells (C) was calculated based on cpm associated with the endothelial cells relative to the total cpm added per well. Shown are means and SD of 5 experiments.]

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by RT-PCR after the 5-h incubation with TNFα were reduced by up to 4.6-fold (Fig. 1A) and 2.5-fold (Fig. 1B), respectively. When adhesion of the CX-1 cells to the endothelial cells was measured, we found that the incremental increase in adhesion attributable to TNFα-induced E-selectin expression was reduced in an ASO dose-dependent manner and abolished at a concentration of 100 nM (Fig. 1C). Control oligonucleotides had no effect on E-selectin expression and tumor cell adhesion.

CX-1 but not MIP-101 Cells Induce Cytokine and E-Selectin Expression on Injection into the Hepatic Circulation. Previously we have shown that highly metastatic murine carcinoma H-59 cells rapidly induce cytokine and E-selectin expression on intrasplenic/portal inoculation in syngeneic mice. Here, we tested whether human colorectal carcinoma cells that are highly metastatic to the liver can induce a similar host cytokine response when xenotransplanted into mice. Results in Fig. 2 show that after the intrasplenic/portal injection of highly metastatic CX-1 cells, there was a rapid increase in hepatic TNFα (Fig. 2A) and IL-1β (Fig. 2B) mRNA expression. This increase was first detectable at 30 min, reached 10-fold relative to control levels at 4 h, and remained high for up to 48 h after tumor inoculation. The increase in cytokine expression was followed by an increase in E-selectin mRNA expression (Fig. 2C), which was measurable at 1 h, reached maximal levels at 4 h, and remained high for 48 h after tumor cell inoculation. Similar to our findings based on the mouse tumor model, the injection of the nonmetastatic colorectal carcinoma MIP-101 cells failed to trigger a cytokine response or E-selectin expression for up to 48 h after tumor cell injection (Fig. 2A–C). A similar induction of E-selectin by CX-1 cells was subsequently confirmed in athymic nude mice (Fig. 2D).

Reduction in Tumor-induced Hepatic E-Selectin Expression after Treatment with C-raf ASOs. To determine whether treatment with C-raf ASO could inhibit tumor-induced hepatic E-selectin expression, the CX-1 cells were injected into nude mice pretreated with C-raf ASO, 24 and 4 h before tumor cell inoculation. Livers were harvested 4 h after tumor cell inoculation, and E-selectin mRNA levels were analyzed using RT-PCR and real time RT-PCR. We found that the injection of C-raf antisense, but not of control oligonucleotides, abrogated tumor-induced E-selectin expression (Fig. 3).

Treatment with C-raf ASOs Inhibits Experimental Liver Metastasis of CX-1 Cells. To investigate whether reduced E-selectin expression in C-raf ASO-treated mice altered the course of experimental liver metastasis, nude mice were tail-vein inoculated with C-raf antisense or control oligonucleotides 24 and 4 h before, as well as 4 h after, the intrasplenic/portal injection of 2 × 10⁶ CX-1 cells. Maintenance oligonucleotide injections were administered once weekly from day 3 onward until the end of the experiment, 4–5 weeks later. In three in vivo experiments performed, the number of metastases in C-raf antisense ASO-treated mice was significantly reduced relative to vehicle or control oligonucleotide-treated animals, whereas no significant difference was observed between the number of metastases in the two control groups. Results of pooled data from 3 experiments (bottom) and livers from control and C-raf ASO treated mice in a representative experiment (top) are shown in Fig. 4A. The median number of metastases based on the pooled data from these
experiments mice was 24 (range, 3–100; n = 11) in vehicle-treated mice; in control oligonucleotides-treated mice, it was 44 (range, 14–100; n = 13); and in C-raf ASO-treated mice, it was 6 (range, 2–21; n = 20) representing an 86% reduction in the number of metastases relative to control oligonucleotide treated mice (P < 0.01). MTT assays showed that C-raf ASO had no direct deleterious effect on CX-1 cell growth at concentrations that were either equal to, or higher than, those used to block endothelial E-selectin expression (Fig. 4B).

Discussion

Our earlier studies have shown that in response to tumor cell entry into the hepatic circulation, a host cytokine response is activated which culminates in the induction of sinusoidal endothelial cell adhesion molecules. E-selectin was the first adhesion molecule to be induced, and it was followed by VCAM-1 and ICAM-1 up-regulation (11). In the present study, we asked whether disruption of the host hepatic inflammatory response to infiltrating colorectal carcinoma cells by C-raf ASO (13) could alter the course of liver colonization. To rule out any direct effect of C-raf down-regulation on tumor cell growth (18) in this study, we used a rodent-specific C-raf antisense sequence with no homology to the human sequence that was previously shown to have no effect on C-raf expression in human cells (14). An MTT assay also confirmed that the ASO had no deleterious effect on human carcinoma CX-1 cell growth in vitro, when tested at concentrations that were effective in blocking C-raf expression in mouse endothelial cells. We show here that the inhibition of the tumor-induced activation of host E-selectin expression resulted in a marked reduction in the number of experimental liver metastases.

In addition to the Ras/Raf/MAPK pathway, other signaling mechanisms involving the p38 MAPK and NFκB were implicated in E-selectin induction by TNFα (10). Previous results have shown that, whereas ERK and JNK activation by TNFα was blocked in cells treated with C-raf ASOs, p38 MAPK activity was unaffected (13). The results imply, therefore, that in hepatic endothelial cells, the inhibition of Raf signaling was sufficient to inhibit E-selectin induction. It should also be noted in this context that the Ras/Raf/MAPK pathway was also implicated in the regulation of outside-in signaling by ligand-activated E-selectin (19). A reduction in C-raf expression in hepatic endothelial cells in vivo may, therefore, have the dual effect of reducing E-selectin expression levels on one hand, and inhibiting the transmission of the E-selectin signal required for tumor cell transmigration and metastasis, on the other (20). The inhibition of other TNFα-dependent mechanisms such as the production of proangiogenic factors may also contribute to reduced metastases formation in the treated mice (21). Consistent with our findings is a recent report that Cimetidine, a histamine type 2 receptor (H2R) antagonist, known to improve the survival of colorectal cancer patients, blocks colorectal carcinoma HT-29 cell adhesion to human umbilical vascular endothelial cell and liver metastasis in nude mice by preventing E-selectin induction (22, 23).

We recently identified the Kupffer cells as the main source of hepatic TNFα in response to inoculation of murine carcinoma H-59 cells. Other studies have shown that Kupffer cells can be activated to release cytokines by colon carcinoma cell-derived carcinoembryonic antigen, and this may be the mechanism of activation by the CX-1 cells (24, 25). Although the C-raf/MAPK pathway was previously implicated in TNFα production by lipopolysaccharide-activated monocytes and macrophages (26), we found that C-raf ASO treatment reduced hepatic TNFα mRNA levels by only 25% relative to controls and did not alter IL-1β levels (data not shown). This suggests that, in the present model, the main impact of the treatment was the inhibition of E-selectin expression, whereas the reduction in TNFα levels may have played an additional, more minor role.

In a previous study, we have shown that tumor cell-induced E-selectin expression was followed 6–8 h later by the appearance of ICAM-1 and VCAM-1, but not by platelet/endothelial cell adhesion molecule (PECAM) mRNA transcripts (11). Treatment with C-raf ASO was shown elsewhere to have only a minor effect on ICAM-1 expression (13). However, we cannot rule out the possibility that, in our model, VCAM-1 expression was also affected, possibly because of a combined effect of the ASO treatment and the inhibition of E-selectin mediated adhesion (5). This may also have contributed to the reduction in metastasis.

In addition to up-regulation of vascular endothelial cell adhesion receptors, the host inflammatory response can facilitate the process of metastasis by other mechanisms including the induction of proangiogenic factors and the release or activation of extracellular matrix-degrading enzymes (21, 27). The present study suggests that effective inhibition of TNFα-induced responses during the very early stages of metastasis can block disease progression. Taken together with other reports, they provide a rationale for developing reagents that target the host inflammatory response for ant metastatic therapy.

5 Abdel-Majid Khatib, Maria Kontogiannia, Lucia Fallavollita, Sarkis Metersissian, Bruce Jamison, Danuta Radzioch, and Pnina Brodt. Increased production of inflammatory cytokines by Kupffer cells in response to metastatic tumor cells, manuscript in preparation.
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References


Fig. 4. A, C-raf ASOs inhibit CX-1 liver metastases formation but do not affect CX-1 proliferation. Nude mice received tail vein injections of C-raf antisense or control oligonucleotides 24 and 4 h before, and 4 h after the intrasplenic/portal injection of 2 × 10⁶ CX-1 cells. Weekly maintenance injections were administered from day 3 onward until the end of the experiment. A second control group was given injections with vehicle (saline) only. A, the results of one representative experiment of three performed. In this experiment, saline-injected mice were killed 34 days and oligonucleotide-treated mice (livers shown) were killed 37 days after tumor inoculation. B, CX-1 cells were incubated with 200 or 400 nM oligonucleotides for up to 72 h. The MTT assay was performed at the indicated intervals. Absorbance measurements were at 540 nm. Shown are the results of a representative experiment of three performed, each in triplicates. Results are expressed as means of triplicate samples. There was no significant difference between the absorbancy readings for the three treatment groups (P > 0.05) at any of the time points analyzed.

A

Control Oligo

C-raf ASO

Metastases/liver

Median and (range)

(3-100)

(14-100)

(2-21)

Untreated

Control Oligo

C-raf ASO

B

OD

24

48

72

200 nM

400 nM

200 nM

400 nM

400 nM
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