Rapid Induction of Cytokine and E-Selectin Expression in the Liver in Response to Metastatic Tumor Cells

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ABSTRACT

The cytokine-inducible endothelial cell adhesion receptor E-selectin has been implicated in cancer metastasis. Previously, we reported that experimental liver metastasis of Lewis lung carcinoma subline H-59 cells could be abrogated in animals treated with an anti-E-selectin antibody. To gain further insight into the functional relevance of E-selectin expression to liver colonization, we investigated here the time course of cytokine and hepatic E-selectin expression after the intrasplenic/portal inoculation of H-59 cells by using a combination of reverse transcription-PCR, Northern blot analysis, immunohistochemistry, and in situ hybridization. In parallel, we analyzed cytokine induction in response to the injection of Lewis lung carcinoma subline M-27 and murine melanoma B16-F1 cells, which do not spontaneously metastasize to the liver. In livers derived from normal or saline-injected mice, only minimal basal levels of TNF-α and IL-1 mRNA were detectable by RT-PCR. Rapid cytokine mRNA induction was noted within 30–60 min of H-59 injection, reaching maximal levels at 4–6 h. This was followed by the appearance of E-selectin mRNA, which was detectable at 2 h after injection and reached maximal levels at 6–8 h, declining to basal levels by 24 h. In situ hybridization analysis and immunohistochemistry localized E-selectin mRNA and protein, respectively, to the sinusoidal endothelium. M-27 cells failed to induce cytokine or E-selectin expression, whereas B-16 cells elicited a delayed and more short-lived response. The results demonstrate that upon entry into the hepatic circulation, tumor cells can rapidly trigger a molecular cascade leading to the induction of E-selectin expression on the sinusoidal endothelium and suggest that E-selectin induction may contribute to the liver-colonizing potential of tumor cells.

INTRODUCTION

The ability of disseminated cancer cells to establish metastases in secondary organs is regulated by a combination of factors including access to the organ microvasculature (hemodynamic factors) and specific host-tumor cell interactions (reviewed in Ref. 1). The attachment of circulating tumor cells to the vascular endothelium of the target organ is thought to be one key step in the metastatic cascade (2). The evidence suggests that this attachment precedes and is required for tumor cell extravasation and subsequent invasion into the target organ parenchyma. Recent studies have identified organ-specific receptors on the luminal surface of the microvascular endothelium that are specifically recognized by tumor cell ligands, thereby facilitating tumor cell arrest and transmigration into the extravascular space (3, 4). Studies of leukocyte transmigration have suggested that the specificity of the interaction between circulating leukocytes and the microvascular endothelium may be determined by the outcome of a series of sequential receptor-ligand interactions rather than the complementarity of a single receptor-ligand system (5). Similar mechanisms are likely to be involved in tumor cell extravasation (reviewed in Ref. 1; Ref. 6).

Prominent among the vascular endothelial cell receptors implicated in leukocyte transmigration are members of the selectin family, i.e., P-, E-, and L-selectin (7). The genes for all three selectins are located on human and murine chromosome 1 (8). These integral membrane proteins are composed of an NH2-terminal C-type lectin domain, an epidermal growth factor domain, variable numbers of complement repeats, a transmembrane domain, and a cytoplasmic tail (9). S-Lewx, a sialylated fucosylated tetrasaccharide and related carbohydrate structures, have been identified as selectin ligands (10). Interestingly, S-Lewx and S-Lewα have also been identified as markers of progression in several types of carcinomas, particularly carcinomas of the gastrointestinal tract, which commonly metastasize to the liver (11, 12). Adhesion studies in vitro have shown that colorectal and other carcinoma cells use S-Lewx and related carbohydrate determinants to adhere to TNF-α-inducible E-selectin on cultured vascular endothelial cells (13–16).

The liver is the primary site of metastasis for some of the most common human malignancies. Liver metastases are frequently inoperable and are associated with poor prognosis (17). We have used subline H-59 of the murine Lewis lung carcinoma to study host-tumor interactions, which regulate liver colonization. Recently, we found that an anti-E-selectin monoclonal antibody, 9A9, when administered to animals in conjunction with these tumor cells, inhibited liver metastases formation (16). Because this effect did not require prior activation of E-selectin by exogenously administered cytokines, we postulated that E-selectin expression was up-regulated locally by endogenous cytokines induced in response to tumor cell inoculation. The objective of the present study was to investigate the course of cytokine and E-selectin expression after the intrahepatic inoculation of tumor cells.

MATERIALS AND METHODS

Cells. The origin and metastatic properties of Lewis lung carcinoma sublines H-59 and M-27 cells were described in detail elsewhere (18–20). H-59 cells are highly metastatic to the liver, regardless of the route of inoculation, whereas M-27 cells metastasize preferentially to the lung. The tumor cells were maintained in vivo by s. c. implantation of liver and lung metastases, respectively, into new recipient animals. Single-cell suspensions of the tumors were obtained by enzymatic digestion of the solid tumors in a 0.02% trypsin solution, and the cells were cultured for 2–4 weeks before their use in the experiments (16). B16-F1 melanoma cells (21) were a kind gift from Dr. A. Chambers (London Regional Cancer Center, London, Ontario, Canada).

Intrasplenic/Portal Injections. Mice were anesthetized by an i.m. injection of 0.5 mg/kg Acepromazine, followed 10–25 min later by an i.m. injection of ketamine/xylazine at final concentrations of 100 and 10 mg/kg, respectively. The spleens were exposed through a small abdominal incision, 10⁶ tumor cells in 0.1 ml saline or saline alone were inoculated, and the animals were splenectomized 1 min later. The livers were removed at different intervals ranging from 30 min to 7 days after tumor inoculation. The liver fragments
were snap-frozen in liquid N₂ and then either stored at −80°C until used for RNA extraction, fixed overnight in 4% paraformaldehyde-PBS at 4°C, and incubated overnight in 5% sucrose-PBS at 4°C for immunohistochemistry or dehydrated and embedded in paraffin by standard techniques for in situ hybridization analysis.

**RT-PCR.** Total cellular RNA was extracted from snap-frozen liver fragments using the Trizol reagent (Life Technologies, Inc., Burlington, Ontario, Canada) according to the manufacturer’s instructions. Two µg of total RNA were reverse transcribed using a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 30 mM KCl, 8 mM MgCl₂, 10 mM DTT, 100 ng oligo(dT)₁₂₋₁₅, 40 units of RNase inhibitor, 1 mM deoxynucleotide triphosphates, and 8 units of avian myeloblastosis virus reverse transcriptase (all from Pharmacia Biotech, Baie D’Urfe, Quebec, Canada). The mixture was incubated for 10 min at 25°C, then for 45 min at 42°C, and finally for 5 min at 95°C. The upstream and downstream primers used were: for IL-1, 5’-CAGATTGCAACTGTTCTGGGACG-3’ and 5’-AAATCTGTCATAGAGGCGATGCC-3’ (Ref. 22; expected product, 233 bp); for TNF-α, 5’-GCAAGTCTCTTTGGTGAGTAAGTTCTCCAGTAGT-3’ and 5’-TCCCTTTGCGAAGCTACGAAGATTG-3’ (23; expected product, 321 bp); for E-selectin, 5’-GTCCGGTGACTGGCTTCCCC-3’ and 5’-GACCTTAGGGTGATTCCCTTG-3’ (24; expected product, 174 bp); and for GAPDH, 5’-GGTGAAGTGCGGTGAACGGATTT-3’ and 5’-AACTGCAAATGGTAGCTGGAATGG-3’ (25; expected product, 520 bp). The cDNA amplification was performed in 25-µl reactions sets by using established procedures with slight modifications (26). Twenty-five cycles consisting of 15 s at 94°C, 15 s at 58°C, and 30 s at 72°C were used, and this was followed by a 10-min incubation at 72°C. The amplified DNA fragments were analyzed without further purification by electrophoresis on a 1% agarose gel.

**Northern Blot Analysis.** The E-selectin cDNA fragment obtained by RT-PCR was purified from agarose gel using the QIAEX Gel extraction kit (Qiagen, Chatsworth, CA), cloned into the pMOSBlue AT-vector (Amerham, Arlington Heights, IL) using the EcoRI cloning site, and used as a probe. Forty µg of total cellular RNA, prepared from frozen liver fragments, were loaded onto a 1.1% agarose gel containing 2 M of formaldehyde, transferred to a nylon membrane (Hybond-N; Amersham) by capillary action, and hybridized with the E-selectin cDNA probe (714 bp), which was radiolabeled by random primer extension with [a-32P]dCTP. Hybridization conditions were as described previously (16). The blots were exposed for 2–6 days at −20°C. As a control for loading, the blots were subsequently probed with a 32 P-labeled, 250-bp cDNA fragment of murine GAPDH (25). The relative amounts of the mRNA transcripts were analyzed by laser densitometry.

**Riboprobe Synthesis and in Situ Hybridization.** Sense and antisense RNAs were synthesized from E-selectin cDNA cloned in the pMOSBlue AT-vector using the T3 and T7 RNA polymerases, respectively. During transcription, DIG-UTP was incorporated into the mRNA product using the DIG RNA labeling kit as instructed by the manufacturer (Boehringer Mannhein, Laval, Quebec, Canada). Paraffin sections (5–10 µm) were prepared from paraformaldehyde-fixed tissue. The mRNA was hybridized with the DIG-labeled antisense RNA, which was then detected by a peroxidase-conjugated anti-DIG antibody used at a dilution of 1:100. A sense RNA was used as a control. The substrate diaminobenzidine was incubated with the sections overnight at room temperature, in the dark. The sections were counterstained with Fast Red.

**Immunohistochemistry.** The paraformaldehyde-fixed, 5% sucrose-treated liver fragments were dried and incubated for 1–2 hours at 4°C in PBS containing 0.1% Tween (PBT) and 2% BSA. Twenty-µm sections prepared from the fragments were incubated for 20 min at room temperature in PBS containing 2% normal donkey serum and then either for 18 h with rat monoclonal antibody to murine E-selectin (Pharmigen, Mississauga, Ontario, Canada) diluted 1:500 or for 1 h with polyclonal rabbit antibodies to murine factor VIII (Dako Corp., Carpinteria, CA), IL-1-α, and TNF-α (Genzyme, Cambridge, MA), all diluted 1:100 in PBT containing 2% BSA. The sections were washed three times in PBT and incubated for 3 h at room temperature either with a CY3-conjugated donkey-anti-rabbit IgG diluted 1:500 for detection of E-selectin or with an FITC-conjugated goat-anti-rabbit IgG (both from Jackson Immunoresearch Laboratories, West Grove, PA) for detection of factor VIII, IL-1-α, and TNF-α. After washing, the sections were mounted in 10% glycerol and examined using a Zeiss Axioshot epifluorescence microscope with 450–490 and 510–560 excitation wavelengths for the FITC and CY3 fluorochromes, respectively.

**RESULTS**

**Induction of IL-1 and TNF-α Expression by Tumor Cells.** To determine whether the injection of tumor cells caused changes in the local cytokine production in the liver, H-59 cells were injected into syngeneic mice by the intrasplenic/portal route, the livers were removed at different time intervals thereafter, and IL-1 and TNF-α mRNA levels were analyzed by RT-PCR. Low constitutive levels of IL-1 (Fig. 1A) and TNF-α (Fig. 1B) mRNA were detected in livers obtained from control, uninjected, or saline-injected animals. mRNA transcripts for both cytokines began to increase within 30–60 min, reaching maximal levels at 4–6 h and declining to basal levels 24 h later. Increased cytokine expression was also noted after the injection of B16-F1 cells, but the increase was seen only at 4 (IL-1) or 6 (TNF-α) h after tumor cell injection, with levels progressively declining after 8 h. The injection of M-27 cells had no effect on the basal IL-1 and TNF-α mRNA expression at any of the time intervals examined (Fig. 1).

**Induction of E-Selectin Expression by Tumor Cells.** E-selectin mRNA expression was also analyzed by RT-PCR. In livers derived from uninjected or from saline-injected mice, no mRNA transcripts were detectable. TNF-α inoculation caused a rapid but short-lived induction of E-selectin (maximal levels at 1 h). In comparison, the injection of H-59 cells caused a slower but sustained increase in E-selectin mRNA levels. These levels began to rise within 2 h, were maximal at 6 h, and returned to basal levels by 34 h after tumor injection. An increase in E-selectin mRNA expression was also noted after injection of B16-F1 cells, but in accordance with the kinetics of cytokine induction, it was detectable only at 4 h after tumor injection, peaking at 8 h and returning to basal levels by 24 h. Consistent with the results of cytokine analyses, the injection of M-27 cells failed to alter E-selectin mRNA levels at any of the time intervals investigated (Fig. 2).

Northern blot analysis confirmed the RT-PCR findings. As shown in Fig. 3, E-selectin mRNA levels increased by 2.7 and 7-fold relative to controls at 4 and 6 h, respectively, after the inoculation of H-59 cells. The levels began to decline at 8 h.

**Localization of E-Selectin mRNA to Hepatic Sinusoidal Endothelial Cells by in Situ Hybridization.** In situ hybridization was used to localize the E-selectin mRNA expressed in response to tumor cell injection. As shown in Fig. 4, the E-selectin signal was associated with the hepatic sinusoidal vessels. These vessels were unstained in livers derived from uninjected or saline-injected mice (Fig. 4B). Control sections labeled with the sense riboprobe were also negative (not shown).

**Immunohistochemical Analysis of Cytokine and E-Selectin Expression.** To analyze changes in hepatic IL-1, TNF-α, and E-selectin protein synthesis in response to tumor cell injection, as shown in Fig. 4, the E-selectin signal was associated with the hepatic sinusoidal vessels. These vessels were unstained in livers derived from uninjected or saline-injected mice, with minimal basal staining for the cytokines that was detectable (Fig. 5C). In contrast, liver cryostat sections obtained from tumor H-59-injected mice, showed intense positive staining within or around sinusoidal vessels for both TNF-α (Fig. 5A) and IL-1 (Fig. 5B) at 6–8 h after tumor inoculation. No E-selectin staining was detectable in livers derived from uninjected or saline-injected mice at any of the time points investigated. In mice injected with H-59 cells, intense staining was seen within 6 h of the injection, and it was associated with the sinusoidal wall as outlined by factor...
VIII staining (Fig. 5F). E-selectin expression declined to background levels by 48 h.

DISCUSSION

The major objective of this study was to investigate early molecular events in the process of liver colonization that are triggered by tumor cell entry into the liver microvasculature and could subsequently determine the course of the metastatic process. Our previous findings with highly metastatic, liver-homing Lewis lung carcinoma subline H-59 cells strongly suggested that hepatic endothelial E-selectin is expressed shortly after tumor cell entry into the liver and that it plays a crucial role in liver metastases formation (16). This implied that an endogenous, local mechanism exists for rapid activation of E-selectin expression by incoming tumor cells. Indeed, the present results show that the intrasplenic/portal inoculation of H-59 cells triggered a rapid increase in hepatic IL-1 and TNF-α mRNA levels. This was followed by rapid induction of E-selectin expression in sinusoidal endothelial cells, which was evident as early as 2 h after tumor injection and persisted for 24 h. This response was tumor type specific because it was either completely absent or delayed after injection of M-27 and B-16 cells, respectively. This correlation between the high liver-colonizing potential of H-59 cells and their ability to induce an early and sustained cytokine response suggests that the host response can affect liver colonization in this model.

The time course for E-selectin expression after tumor cell injection differed from that after direct cytokine inoculation, the latter being more rapid (maximal mRNA induction by 1 h) and short lived (signal undetectable by 2 h). This delay in the tumor-induced response suggests that tumor cells activate a resident hepatic cell population to release the cytokines, which in turn up-regulate E-selectin expression. Although the cellular source of these cytokines in the liver remains to be positively identified, several cell types could conceivably be involved. They include the sinusoidal Kupffer cells (27), circulating cells such as polymorphonuclear leukocytes or platelets (28), and possibly the endothelial cells themselves (29). Indeed, immunohistochemistry results suggest that IL-1 and TNF-α were produced by cells localized in, or adjacent to, the hepatic sinusoids. These cells could have responded to tissue damage caused by the influx of tumor cells (30) or to soluble mediators such as cytokines or chemokines released by the tumor cells (31). Analysis by the ELISA failed to detect IL-1
and TNF-α in medium conditioned by H-59 cells (data not shown). However, the possibility that one or both of these cytokines are produced by the tumor cells in the liver, in response to a local trigger, cannot at present be ruled out.

Of the tumor lines used in the present study, only H-59 and B-16 cells could induce cytokine and E-selectin expression in the hepatic microenvironment, and the time course of the responses induced by these cell types were distinct. The reasons for this divergence in the host response are not presently clear, but several mechanisms may be postulated. Differences in the ability of the tumor cells to produce and/or induce other cells to release soluble mediators such as NO, prostaglandin E2, and IFN-γ may be a contributing factor. These mediators, either alone or in combination, are potent inducers of cytokines such as IL-1 and TNF-α (32–34) and can be produced by a variety of cell types including hepatocytes (35), macrophages (36), Kupffer cells (37, 38), hepatic sinusoidal endothelial cells (39), and tumor cells (40–41). They have been implicated in both positive and negative regulation of the metastatic phenotype in various tumor types (42–44), a role that may be related to their ability to regulate the expression of adhesion molecules (45–48). Alternatively, the divergent potentials of the tumor cells to activate E-selectin expression may reflect differences in the proportion, location, and/or survival time of tumor cells arresting in the hepatic microvasculature (49). These, in turn could be determined by cell size and deformability (50) or may be a function of cell ‘‘stickiness’’ and the ability to attach to sinusoidal cells such as Kupffer cells. Attachment to Kupffer cells was, in fact, shown to correlate with the liver-colonizing potential in other...
tumor models (51), and it may determine tumor cell ability to elicit cytokine production by these cells.

B-16-F1 cells are poorly metastatic to the liver, but their liver-colonizing potential was shown recently by Sherbarth and Orr to be significantly augmented after pretreatment of recipient animals with IL-1\(\alpha\) (49). This effect was attributed to the induction of vascular endothelial adhesion receptors in response to the cytokine (49). These results and other studies by Araki et al. (52) suggest that preextravasation events play a role in liver colonization by the B-16 cells. This implies that the delayed and short-lived E-selectin up-regulation, which we observed in response to B16-F1 inoculation, may have functional consequences in respect to the ability of these cells to form hepatic metastases. On the other hand, recent findings by Luzzi et al. (53) that the majority of intrahepatically injected B16-F1 cells cannot survive to form metastases, even after extravasation, argue that transmigration into the extravascular space is only one of multiple factors limiting the ability of these cells to colonize the liver.

In addition to E-selectin, we found that the injection of H-59 cells caused an increase in the expression of hepatic P-selectin and subsequently the induction of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (5). B16-F1 cells induced P-selectin, a weak vascular cell adhesion molecule 1 and no intercellular adhesion molecule-1 expression, whereas M-27 cells failed to induce changes in the levels of any of these adhesion molecules.\(^4\) These results suggest that similarly to molecular events occurring during leukocytes migration (5), tumor cell adhesion to sinusoidal endothelial E-selectin may be the initial critical event which triggers the expression of other endothelial cells receptors and sets in motion the adhesion cascade necessary for transendothelial migration of the tumor cells.

Taken together with our previous studies (16), the present results suggest that reagents that can interfere with hepatic E-selectin induction and/or function could potentially have therapeutic, antimetastatic effects during the early stages of liver colonization.

REFERENCES


\(^4\) A-M. Khatib and P. Brodt, unpublished observation.


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