Role of Carcinoembryonic Antigen in the Progression of Colon Cancer Cells That Express Carbohydrate Antigen

Shigeki Minami,1 Junichiro Furui, and Takashi Kanematsu
Department of Surgery II, Nagasaki University School of Medicine, Nagasaki 852-8501, Japan

ABSTRACT

Carcinoembryonic antigen (CEA) has been reported to promote the metastatic potential in some experimental tumors. Adhesion molecules are known to play an important role in the process of metastasis. Cytokines, including interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α), which are produced by Kupffer cells, induce endothelial cells to express adhesion molecules. As a result, the present study was designed to investigate whether the interaction between CEA and Kupffer cells accelerated the metastatic potential of tumors in the liver. Kupffer cells isolated from the liver of male BALB/c mice were cultured with CEA, either with or without the addition of a cytokine inhibitor. The levels of IL-1β and TNF-α were examined in a culture medium. An adhesion assay of colon cancer cell lines to human umbilical vein endothelial cells was also performed. When CEA was added to the Kupffer cell culture medium, cytokines were produced. Elevated levels of cytokines appeared to lead to increased rates of adhesion of cancer cells to endothelial cells. However, these phenomena were blocked by the addition of cytokine inhibitors. CEA stimulated Kupffer cells to produce cytokines. An elevated number of cytokines have been proven to promote the expression of adhesion molecules in endothelial cells. These processes are therefore considered to contribute to the metastasis of malignant cells to the liver. These results suggest that cytokine inhibitors may therefore play an important role in the inhibition of hepatic metastasis.

INTRODUCTION

CEA1 is a glycosylated protein that is useful as a tumor marker for predicting recurrence in gastrointestinal malignancies. CEA has been experimentally implicated in the development of hepatic metastasis of human colorectal cancers (1). The i.v. injection of CEA in athymic nude mice, prior to the intrasplenic injection of colorectal cancer cell lines with a low metastatic potential, has been reported to enhance hepatic metastasis (2). In addition, metastatic human colon cancer cell lines have also been shown to acquire a highly metastatic potential when transfected with the cDNA coding for CEA (3, 4). Although these data suggested that CEA plays a role in the formation of hepatic metastasis, the mechanism by which CEA causes an enhancement of metastasis has yet to be elucidated.

Kupffer cells in the liver are known to bind CEA (5–7). Kupffer cells are the major source of cytokine production in the liver, and these cells secrete such cytokines as IL-1β and TNF-α (8), which are known to induce endothelial cells to express E-selectin. When E-selectin is present on human endothelial cells activated from IL-1β and TNF-α, cancer cells expressing carbohydrate have been reported to easily adhere to endothelial cells (9–11).

The carbohydrate antigens, sLeX and sLeA, are expressed on human colorectal cancer cells (11). The amount of sialic acid present on the surface of malignant cells has been demonstrated to correlate with the ability of these cells to metastasize (12). The metastatic potential of murine tumor cell lines has been shown to be directly proportional to cell surface sialylation (13). These carbohydrate antigens, which are ligands for E-selectin, may thus play an important role in the development of metastasis.

The present study was performed to investigate whether the interaction between CEA and Kupffer cells correlated the adhesion of tumor cells with sLeX and/or sLeA to endothelial cells.

MATERIALS AND METHODS

Reagents. CEA and lipopolysaccharide were purchased from the Sigma Chemical Co. (St. Louis, MO). FR167653 was purchased from the Fujisawa Pharmaceutical Co. Ltd. (Tokyo, Japan). FR167653 is a low molecular weight inflammatory cytokine inhibitor that inhibits the production of IL-1α, IL-1β, and TNF-α (14).

Kupffer Cell Isolation and the in Vitro Activation of Kupffer Cells. Kupffer cells were isolated from male BALB/c mice according to a protocol of collagenase infusion, followed by metrizamide density gradient separation with a selective adherence to plastic (8). The Kupffer cells were cultured in serum-free RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) with or without 1 × 106 to 1 × 107 ng/ml of CEA and also with or without 1 × 102 to 1 × 103 ng/ml of FR167653. This concentration of FR167653 inhibited the production of these cytokines without cytotoxicity for mouse Kupffer cells (data not shown). This CEA concentration was instituted because we think that the CEA concentration may increase at the local hepatic sinusoid.

Cytokine Assay for ELISA. The spent cell culture medium was collected and assayed for IL-1β and TNF-α using a commercially available mouse cytokine ELISA kit (BioSource International, Inc., Camarillo, CA). The cytokine expression was plotted as pg/ml after determining the values obtained from the untreated control group.

Detection of IL-1β and TNF-α mRNA Expression Using Reverse Transcription-PCR. The total RNA from the harvest cells was isolated by the acid guanidium thiocyanate-phenol-chloroform method (15), and 3 µg of total RNA were converted to cDNA by reverse transcription using the SuperScript pre-amplification system (Life Technologies, Inc., Grand Island, NY). The amplification procedure consisted of 30 cycles as determined previously (16). These sequences corresponded to IL-1β and TNF-α cDNA from the BALB/c mouse Kupffer cells. The sequences corresponding to the published mouse β-actin cDNA sequence were also used as controls (16). The amplified DNA fragments were as expected, 515 bp for IL-1β, 400 bp for TNF-α, and 400 bp for β-actin. Each final PCR product, 10 µl, was loaded onto a composite gel containing 2% agarose (FMC Co., Rockland, ME), electrophoresed, and visualized by ethidium bromide staining.

Human Cancer Cell Lines. The cultured human cancer cells, CaR-1 and COLO 201, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum. A significant contribution of the sLeX antigen was noted in the adhesion of CaR-1. On the other hand, COLO 201 exhibited sLeA-dependent adhesion (11).

Antibodies. Antimouse monoclonal antibody against E-selectin was obtained from Becton Dickinson (San Jose, CA). Nonspecific staining was assessed in parallel by adding antimouse monoclonal IgG control antibodies (Dako A/S, Tokyo, Japan).

Immunohistochemistry. HUVECs were cultured for 40 min with each spent Kupffer cell culture medium on a LabTek chamber slide (Nalge Nunc International, Naperville, IL), fixed in acetone at 4°C for 10 min, and then overlaid for 2 h at room temperature with anti-E-selectin antibody. After several washes, the cells were incubated for 30 min with peroxidase-conju-

Received 8/21/00; accepted 1/17/01.
1 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
2 To whom requests for reprints should be addressed, at Department of Surgery II, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
3 The abbreviations used are: CEA, carcinoembryonic antigen; IL, interleukin; TNF, tumor necrosis factor; sLeX, sialyl Lewis X; sLeA, sialyl Lewis A; FR167653, 1-[7-(4-flurophenyl)-1,2,3,4-tetrahydro-8-C4-pyridyl[5,1-c] [1,2,4] triazin-2-yl]-2-phenyl-ethanedione sulfate monohydrate; HUVEC, human umbilical vein endothelial cell.
gated rat antimouse immunoglobulin G (Dako A/S). The slides were stained for peroxidase activity with 0.05%, 3,3'-diaminobenzidine/0.01% H₂O₂.

**Monolayer Cell Adhesion Assay using HUVECs.** An adhesion assay was performed as described previously (11). HUVECs (Kurabou Co. Ltd., Osaka, Japan) were stimulated with each spent Kupffer cell culture medium for 40 min. Next, §Cr-labeled cultured human cancer cells were added to these plates and incubated for 30 min at room temperature with rotation (11). After the adherent cells were washed three times, they were treated with 1% Triton X-100. The lysate was collected, and the radioactivity in each well was measured with a gamma counter. The number of attached cells was calculated from the specific radioactivity of the labeled cells. The adherence of cancer cells was corrected for binding to unstimulated HUVECs.

**Statistical Analysis.** The results are expressed as the mean ± SE, and the groups of means were tested by one-way ANOVA. Differences between the mean values of the different groups were tested by ANOVA, followed by Fisher’s protected least significant difference with a significance level of 5%.

**RESULTS**

**Induction of Cytokine Expression from Kupffer Cells.** A dose-dependent response in the mouse IL-1β expression was evident after treatment with CEA. The IL-1β response in mouse Kupffer cells treated by CEA without FR167653 increased in comparison with the control group (P < 0.01). The CEA with FR167653 showed significantly (P < 0.01) lower responses compared with CEA with an increase in the mouse IL-1β expression (Fig. 1A).

The pattern of mouse TNF-α expression was closely similar to that of the IL-1β response. The mouse TNF-α expression showed a dose-dependent tendency in the CEA-treated group. The TNF-α induction in the CEA-treated groups increased in comparison with the control (P < 0.01). Lower responses of mouse TNF-α in the CEA with FR167653-treated groups were noted in contrast with the significant (P < 0.01) increase in the response observed in the CEA-treated groups (Fig. 1B).

**Induction of Cytokine mRNAs in Kupffer Cells.** The cytokine-specific mRNA obtained from the PCR products demonstrated elevations in both the cytokine transcripts in the CEA-treated groups, when compared with the untreated controls. The level of the IL-1β transcript expression in the CEA-treated groups was 2.0 times greater than that in the CEA with FR167653-treated groups. The expression of the TNF-α transcripts in the CEA-treated groups was 1.5 times greater than that in the CEA with FR167653-treated groups (Fig. 2).

**Expression of E-Selectin.** E-selectin immunostaining was found in the HUVECs pretreated with the spent Kupffer cell culture medium with CEA. In the HUVECs preincubated with the spent Kupffer cell culture medium with CEA and FR167653, no expression of E-selectin was observed (Fig. 3). Also, the expression of E-selectin was not observed in the HUVECs pretreated with CEA alone (data not shown).

**Adhesion of Human Cancer Cells to the HUVECs Activated by Each Spent Kupffer Cell Culture Medium.** The adhesion of cancer cells to the HUVECs was accelerated by the spent culture medium with CEA. The spent Kupffer cell culture medium with CEA and FR167653 showed a clearly inhibitory effect on the adhesion of cancer cells to the HUVECs (Fig. 4). In addition, the adhesion rate of the CEA-treated groups was significantly higher than that of the control group (P < 0.01). The addition of FR167653 therefore inhibited cell adhesion (P < 0.01; Fig. 5). The adhesion was inhibited by antibody to the binding domain of E-selectin and by removing calcium ions with EDTA (data not shown).

**DISCUSSION**

CEA receptor has been shown to be present on the surface of Kupffer cells. In addition, an Mₘ 80,000 CEA-binding protein has been identified in both rodent and human Kupffer cells, which binds and clears soluble CEA from the circulation (5–7). CEA is recognized by this protein through a five-amino acid sequence, Pro-Glu-Leu-Pro-Lys (PELPK), located at the hinge region between the NH₂-terminal and the first immunoglobulin loop domain in the CEA sequence (5). CEA binding and internalization by Kupffer cells are consistent with the mechanism of receptor-mediated endocytosis (17). The binding of CEA to Kupffer cells via the peptide sequence PELPK thus results in the release of a series of cytokines (8).

It is unlikely that CEA is related to cell growth, because neither the introduction of CEA cDNA nor the treatment with anti-CEA Fab changes the growth rate of the tumor cells (3). One possible mechanism of the CEA effect on metastasis was thought to be that metastasis was enhanced because of the homotypic adhesion between the CEA molecules sequestered by Kupffer cells in the liver and CEA on the tumor cell surface. However, this is unlikely because the non-CEA-producing cell lines are also enhancible by the preinjection of CEA.
Another possible mechanism is that the increased metastatic potential to the liver might be related to the increased homotypic binding through CEA (3, 18). The capability of tumor cells to aggregate is associated with the metastatic potential, probably because cell aggregates have a greater chance to be trapped in the microvasculature than single cells.

The present study demonstrated that the culture of mouse Kupffer cells with CEA resulted in the release of IL-1β and TNF-α. E-selectin was expressed on endothelial cells by a spent culture medium of Kupffer cells with CEA. It is therefore possible that the binding of CEA to Kupffer cells may thus influence the transition of sinusoidal endothelial cells. The endothelial cells expressed the E-selectin.

![Figure 2](image2.png)

Fig. 2. The cDNA patterns in isolated mouse Kupffer cells in each group were shown by RT-PCR. The products on the 515-bp band and 400-bp band correspond to IL-1β and TNF-α, respectively. The cytokine-specific mRNA identified elevations in the CEA-treated groups.

![Figure 3](image3.png)

Fig. 3. Immunohistochemical demonstration of E-selectin to cultured HUVECs. The expression of E-selectin was shown in the HUVECs pretreated with the spent Kupffer cell culture medium with CEA (A). FR167653 inhibited the expression of E-selectin (B).

![Figure 4](image4.png)

Fig. 4. Adhesion of cancer cells to the HUVECs. Cancer cells closely adhered to the HUVECs treated with the spent culture medium of mouse Kupffer cells treated by CEA (A). FR167653 inhibited the adhesion of cancer cells to the HUVECs (B).

![Figure 5](image5.png)

Fig. 5. Adhesion rate of cancer cells to the HUVECs treated by the spent culture medium of mouse Kupffer cells. The adhesion of cancer cells to the HUVECs increased after the treatment with CEA but thereafter was inhibited after treatment with FR167653 (*, P < 0.01). Bars, SE.
enhanced adhesion of colorectal carcinoma cells with sialylated Lewis antigens. Indeed, tumor cell-dependent activation of hepatic sinusoid and/or platelets, and subsequent release of factors that activate endothelial cells to elicit E-selectin expression, were observed previously (19, 20). The production of a series of cytokines by the interaction between CEA and Kupffer cells therefore induced sinusoidal endothelial cells to express adhesion molecules and also helped contribute to the formation of hepatic metastasis from colorectal cancer. Cytokine-activated endothelial cells have expressed not only E-selectin but also vascular cell adhesion molecule 1 and intercellular adhesion molecule-1 (21). We therefore think that vascular cell adhesion molecule 1 and intercellular adhesion molecule-1 are likely to remain necessary for further examination in this pathway. In addition, we evaluated in vitro FR167653, a dual inhibitor of IL-1 and TNF-α, and found it to inhibit the production of a series of cytokines from mouse Kupffer cells.

In conclusion, CEA was observed to enhance the adhesion of cancer cells and epithelial cells, through the production of cytokines by the interaction between CEA and Kupffer cells. The inhibition of these cytokines is thus considered to be necessary to prevent the development of hepatic metastasis, and cytokine inhibitors, such as FR167653, may therefore play a useful role in the inhibition of hepatic metastasis.

REFERENCES

Role of Carcinoembryonic Antigen in the Progression of Colon Cancer Cells That Express Carbohydrate Antigen

Shigeki Minami, Junichiro Furui and Takashi Kanematsu

Cancer Res 2001;61:2732-2735.