

Essential Roles of Tumor Necrosis Factor Receptor p55 in Liver Metastasis of Intrasplenic Administration of Colon 26 Cells

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ABSTRACT

Intrasplenic administration of a colon adenocarcinoma cell line, colon 26, induced tumor necrosis factor (TNF) α protein expression around the central and portal veins of the liver at 3 days, and liver metastases by 24 days after the tumor injection, in 90% of wild-type (WT) mice. To explore the roles of TNF- α in the process, we administered colon 26 cells into tumor necrosis factor receptor p55 (TNF-Rp55) knockout (KO) mice. Less than 50% of TNF-Rp55 KO mice developed liver metastasis with significantly lower liver weights and the volumes of metastatic foci. These observations suggest the critical roles of TNF-Rp55-mediated signals in this liver metastasis model. The intrasplenic tumor injection induced mRNA expressions of vascular endothelial growth factor, heparin-binding epidermal growth factor, matrix metalloproteinase-9, and tissue inhibitor of matrix metalloproteinase-1 at similar levels in the livers of both WT and TNF-Rp55 KO mice. Immunohistochemical analyses of the livers of WT mice after tumor injection demonstrated the enhanced expression of vascular cell adhesion molecule (VCAM)-1 and E-selectin on sinusoidal endothelial cells. Enhanced E-selectin expression was similarly observed in the liver of TNF-Rp55 KO mice after tumor injection. However, the enhancement in VCAM-1 mRNA expression and its protein production was significantly attenuated in the liver of TNF-Rp55 KO mice when compared with WT mice. Collectively, these observations suggest that TNF-Rp55-mediated signals can up-regulate both VCAM-1 expression in the liver and subsequent liver metastasis after intrasplenic tumor injection.

INTRODUCTION

Despite recent advances in diagnostic and therapeutic measures, the prognosis of cancer patients with metastasis still remains poor. Thus, it is mandatory to elucidate the molecular mechanism of the metastasis process to develop appropriate treatment modalities. Accumulating evidence indicates that progressive tumor growth depends on a series of sequential and highly selective steps, each of which is rate limiting (1). The first step is tumor growth at the primary site, which is supported by neovascularization (1, 2). Subsequently, tumors degrade basement membranes mediated by MMPs² and invade venules or lymphatics (3). Tumor cells then circulate, and those expressing adhesion molecules adhered to capillary beds in distant organs (4). Adherent tumor cells extravasate into tissues by degrading basement membranes with MMPs. Finally, the tumor cells proliferate in distant organs, in association with neovascularization.

TNF- α was originally identified as a cytokine responsible for

endotoxin-induced tumor necrosis (5). Several independent groups reported that therapy with recombinant TNF- α was effective against several types of murine tumor models of hepatic (6, 7) and pulmonary metastasis (8), particularly when it was administered in combination with IFN- γ (9) or IL-2 (10, 11). Moreover, a Phase I clinical study of TNF- α treatment demonstrated partial efficacy against metastatic spread (12, 13). However, in several models, the administration of TNF- α or the TNF- α gene transduction into tumor cells enhanced the incidence of metastasis (14–17). These contradictory results may be explained by differences in the cell types used in each experiment (17). Consequently, the role of endogenous TNF- α in the metastatic process remains to be determined.

TNF- α uses two types of receptors, encoded by distinct genes: a TNF receptor with a molecular weight of M_r 75,000 (TNF-Rp75) and one with a molecular weight of M_r 55,000 (TNF-Rp55; Ref. 18). These two receptors show 30% homology at the amino acid level in their extracellular, cysteine-rich, and ligand-binding regions (19). TNF-Rp75 expression is restricted to hematopoietic and endothelial cells, and mediates a limited number of the biological activities of TNF- α , including thymocyte and T-cell proliferation (20). In contrast, TNF-Rp55 is ubiquitously expressed on almost all cell types except for RBCs, and mediates the various activities of TNF- α .

Therefore, we investigated the role of TNF- α in the spread of tumor metastases. Prior studies have revealed that p55 but not p75 is expressed abundantly in liver.³ Hence, to evaluate the roles of TNF- α , particularly TNF-Rp55-mediated signals, we injected a murine colon adenocarcinoma cell line into the spleen of WT and TNF-Rp55 KO mice. This approach has provided definitive evidence that the absence of TNF-Rp55 results in attenuated VCAM-1 expression in the liver and also reduced the incidence of liver metastasis.

MATERIALS AND METHODS

Mice. Specific pathogen-free 8–12-week old female BALB/c mice were purchased from Charles River Japan Co. (Yokohama, Japan) and designated as WT mice. TNF-Rp55-deficient mice were a kind gift of Dr. Horst Bluethmann (Hoffmann-Roche, Basel, Switzerland; Ref. 21), and were backcrossed to BALB/c mice for >6 generations in our animal facility (22) and designated as TNF-Rp55 KO mice. All of the animal experiments were performed in accordance with the Guideline for the Care and Use of Laboratory Animals in the Takara-machi Campus of Kanazawa University.

Induction of Liver Metastasis. A murine adenocarcinoma cell line, colon 26 (23), was maintained in DMEM with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂. Subconfluent cells were collected and resuspended in HBSS at a cell density of 5×10^5 cells/ml. The cell viability was always >95% by a trypan blue exclusion test. One-hundred μ l of cell suspension was injected into the spleens of mice under light ether anesthesia. Mice were sacrificed at the indicated time intervals after tumor injections to determine the incidence of liver metastasis, the number of metastatic foci in the liver, and the tumor size. Tumor size was calculated according to an equation of $a \times b^2/2$, where a and b indicate long and short diameters of the tumor, respectively (24). In another series of experiments, the livers were removed from the mice at

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² The abbreviations used are: MMP, matrix metalloproteinase; TNF, tumor necrosis factor; IL, interleukin; TNF-R, tumor necrosis factor receptor; VCAM-1, vascular cell adhesion molecule-1; WT, wild-type; KO, knockout; ICAM-1, intercellular adhesion molecule-1; RT-PCR, reverse transcription-PCR; TIMP, tissue inhibitor of matrix metalloproteinase; VLA-4, very late antigen-4.

³ T. Kondo and N. Mukaida, unpublished observations.

Table 1 Sequences of the primers and conditions of PCR

| | Sense primer | Antisense primer | Annealing temperature | Cycles | Product (bp) |
|----------------|------------------------|-------------------------|-----------------------|--------|--------------|
| TNF- α | AGTTCATGGCCAGACCT | CGGACTCCGCAAGTCTAAG | 55°C, 1 min | 32 | 463 |
| IL-1 α | ATGTATGCCTACTCGTCGGG | CAGACTGTCAGCACTTCCCA | 55°C, 1 min | 30 | 453 |
| IL-1 β | GCAGACAACGCACTACAGGCTC | GCTCTGCTTGTGAGGTGCTGATG | 55°C, 1 min | 32 | 376 |
| VCAM-1 | CCCAAGGATCCAGAGATTCA | GTCATTGTCCAGCACCACC | 55°C, 1 min | 35 | 389 |
| E-selectin | AAAACGCGAGAAGAACGGA | TGTTGTTGGTTCACCTGGA | 55°C, 1 min | 40 | 443 |
| β -actin | TGTGATGTTGGGAATGGGTCAG | TTGATGTCACGCACGATTCC | 55°C, 1 min | 25 | 514 |

the indicated time intervals after intrasplenic tumor injections for histological analysis and total RNA extraction.

Antibodies. Rat anti-mouse VCAM-1 and E-selectin monoclonal antibodies were obtained from BD Biosciences/PharMingen (San Diego, CA). Rat monoclonal antibody to a specific marker for macrophages and Kupffer cells, F4/80, was obtained from Serotec (Oxford, United Kingdom). Rabbit anti-mouse TNF- α IgG was prepared as described previously (25). Isotype-matched IgG from the same species was used as a negative control.

Histological, Immunohistochemical, and Immunofluorescence Examination. For a histological examination, a portion of liver tissue was fixed with 10% neutrally buffered formaldehyde and embedded in paraffin. The sections were deparaffinized and stained with a H&E solution. For immunohistochemical analysis, paraffin-embedded sections were deparaffinized and incubated for 30 min with 0.3% H₂O₂ in methanol to delete endogenous peroxidase. Subsequently, endogenous avidin and biotin were blocked with Avidin-Biotin Blocking kit (DAKO, Carpinteria, CA), and nonspecific protein binding was blocked with 10% serum obtained from the same species used to produce the secondary antibody. Thereafter, the sections were incubated with rat anti-VCAM-1 (diluted at 1:100), anti-E-selectin (diluted at 1:100), anti-F4/80 (diluted at 1:20), or rabbit anti-TNF- α (30 μ g/ml) antibodies. After being washed three times with PBS, the sections were incubated for 30 min at room temperature with biotinylated rabbit antirat IgG and biotinylated goat antirabbit IgG to detect F4/80 and TNF- α , respectively. The immune complexes were visualized using ELITE ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The numbers of F4/80- or TNF- α -positive cells were determined on five randomly chosen high-power fields by an examiner without any knowledge of the experimental procedures. To detect VCAM-1 and E-selectin, Catalized Signal Amplification System (DAKO) was used according to the manufacturer's instructions. Positive areas were determined with the use of NIH Image Analysis software (version 1.62; NIH, Bethesda, MD). A double-color immunofluorescence analysis was performed to detect F4/80- and TNF- α -positive cells as described previously (22).

RNA Isolation and Semiquantitative RT-PCR. Total RNAs were isolated from a proportion of liver tissues with RNazol B (Biotex Laboratory Inc., Houston, TX) as described previously (22). Five μ g of total RNA was reverse-transcribed at 42°C for 1 h in a 20- μ l reaction mixture containing mouse Moloney murine leukemia virus reverse transcriptase (Toyobo, Osaka, Japan) and oligodeoxythymidylic acid primers. Thereafter, 0.5 μ l of cDNA products were amplified using sets of primers that specifically amplify mouse TNF- α , IL-1 α , IL-1 β , VCAM-1, E-selectin, or β -actin, under conditions to ensure that the quantity of the amplified products did not reach saturation points (Table 1). The primers were designed based on the data deposited in the Gene Bank and prepared by Genset Oligos (Kyoto, Japan). The amplified PCR products were fractionated on a 1.5% agarose gel and visualized by ethidium bromide. The band intensities were measured with the aid of NIH Image Analysis software, and the ratios to β -actin were determined. The relative intensities were calculated based on the assumption that the ratio of the untreated WT mice was 1.00.

Statistical Analysis. Data were analyzed statistically using one-way ANOVA followed by the Fisher's protected least significance difference test or Mann-Whitney's *t* test. *P*s < 0.05 were considered statistically significant.

RESULTS

Metastasis Formation. Macroscopic liver metastasis started to appear at 19 days after the intrasplenic injection of colon 26 cells in 3 of 4 mice with an intrahepatic tumor volume of 3.5 ± 1.7 mm³. By 24 days after intrasplenic tumor injection, all 4 of the WT mice developed multiple macroscopical metastatic foci in the liver with an

intrahepatic tumor volume of 211.4 ± 33.4 mm³. Therefore, we subsequently determined the incidence of metastasis, the metastatic focus numbers, and the tumor sizes in the liver at 24 days after the injection.

TNF- α Expression at the Site of Liver Metastasis. We next determined whether TNF- α was expressed in the liver during the course of liver metastasis. TNF- α transcripts were rarely detected in untreated WT mice (Fig. 1). Intrasplenic injection of colon 26 cells induced an increase in TNF- α transcripts in the liver of WT mice, starting at 6 h and reaching a maximal level at 24 h. TNF- α mRNA expression levels remained elevated for up to 12 days, when compared with vehicle-injected mice (Fig. 1). Immunohistochemical analysis rarely detected immunoreactive TNF- α proteins in the livers of untreated WT mice (Fig. 2). However, by 3 days after the intrasplenic tumor injection, TNF- α proteins were detected around the central and portal veins (Fig. 2). TNF- α -positive cells were diffusely distributed in the liver of WT mice at 6 days but were decreased by 12 days after intrasplenic tumor injection (Fig. 2). Double color immunofluores-

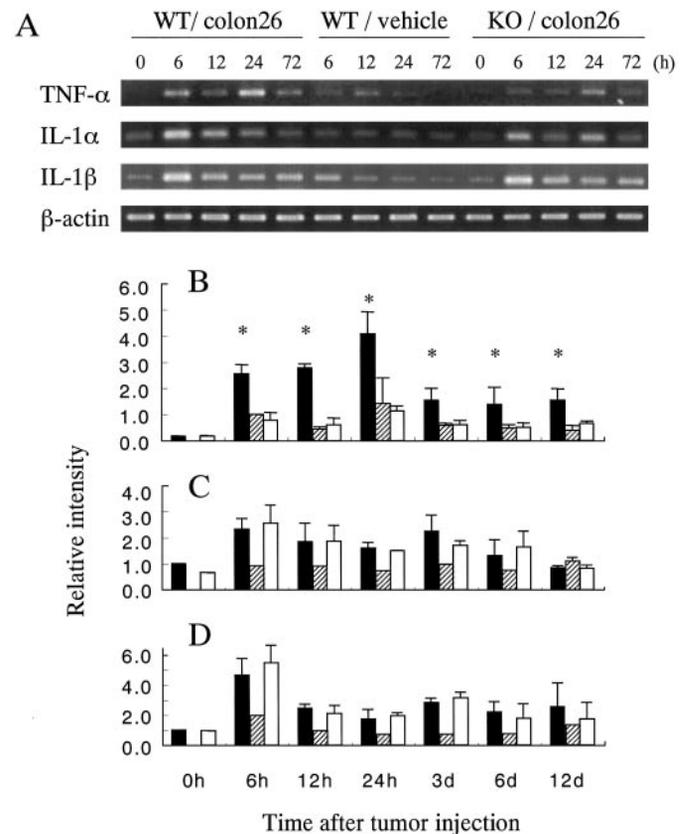


Fig. 1. Cytokine gene expression in the liver. Total RNAs were extracted from the livers of WT and TNF-Rp55 KO mice at the indicated time intervals. RT-PCR analyses were performed, and representative results from three individual experiments are shown here in A. The ratios of TNF- α (B), IL-1 α (C), and IL-1 β (D) to β -actin of the tumor-injected WT mice (■), the tumor-injected TNF-Rp55 KO mice (□), and the vehicle-injected WT mice (▨) were calculated by designating the ratio of the vehicle-treated WT mice 6 h after tumor injection as 1.0; bars, \pm SE. (*n* = 6). *, *P* < 0.05, when compared between WT and TNF-Rp55 KO mice.

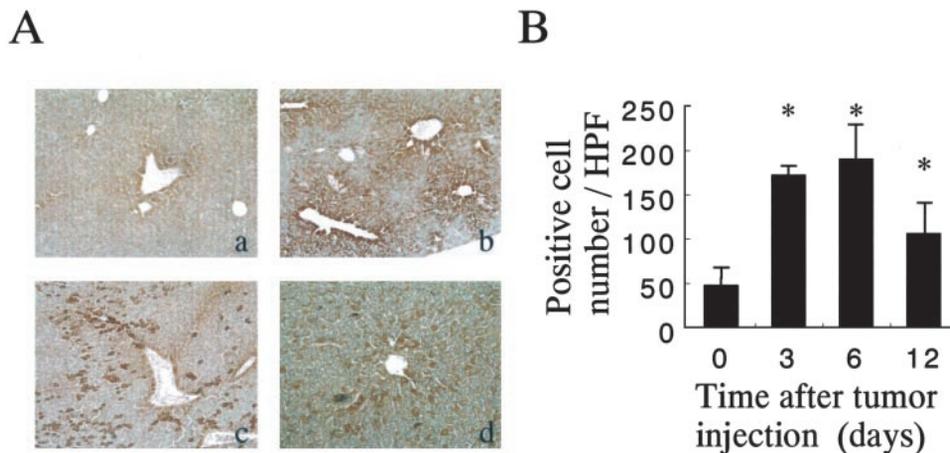


Fig. 2. A, immunohistochemical analysis of TNF- α proteins of liver tissues of WT mice before and after tumor injection (a, day 0; b, day 3; c, day 6; d, day 12). Representative results from at least 3 animals in each group are shown. Original magnification, $\times 200$. B, the number of TNF- α -positive cells in liver tissue from WT mice (■) after tumor injection. All values represent the mean; bars, \pm SE on the results from 6 individual animals. *, $P < 0.05$.

cence analysis demonstrated that immunoreactive TNF- α proteins were restricted to F4/80-positive cells in untreated WT mice (Fig. 3). However, at 3 and 6 days after the injection, when TNF- α production was up-regulated, TNF- α proteins were detected on both F4/80-positive and -negative cells (Fig. 3; data not shown). Most F4/80-negative cells appeared to be hepatocytes based on their morphology. By 12 days after tumor injection, TNF- α proteins were again expressed only by F4/80-positive cells (Fig. 3). These results indicate that TNF- α was produced by F4/80-positive macrophages and Kupffer cells, as well as by F4/80-negative hepatocytes before the appearance of macroscopic metastasis.

Reduced Incidence of Liver Metastasis in TNF-Rp55 KO Mice.

To clarify the pathological roles of intrahepatically produced TNF- α in liver metastasis, we injected colon 26 cells into the spleens of WT and TNF-Rp55 KO mice. At 24 days after the intrasplenic injection of colon 26 cells, there was no statistical significance in sizes of spleens between TNF-Rp55 KO and WT mice (Table 2). These results indicate that the absence of TNF-Rp55 did not affect the local growth of tumors in the injected organ. However, $>90\%$ of WT mice developed metastasis in the liver, whereas $<50\%$ of TNF-Rp55 KO mice developed metastasis in the liver. Moreover, when we determined the

volumes of the intrahepatic metastatic foci and liver weights in mice with liver metastases, both parameters were significantly greater in WT mice than TNF-Rp55 KO mice (Table 2). Thus, the absence of TNF-Rp55 reduced the extent of liver metastasis, but without any discernible effects on the tumor growth at the injected organ site.

Comparison of Expression of mRNA for Angiogenic Factors, MMP, and TIMP by WT and TNF-Rp55 KO Mice. Because neovascularization is a prerequisite for the establishment and growth of metastasis, we investigated the gene expression of potent angiogenic factors (26–28), vascular endothelial growth factor, basic fibroblast growth factor, and heparin-binding epidermal growth factor by RT-PCR. The gene expression of each of these angiogenic factors was enhanced to an equal extent in the livers of WT and TNF-Rp55 KO after intrasplenic tumor injection at any time points that we examined (data not shown). Similar results were obtained for the expression patterns of vascular endothelial growth factor receptors, Flk-1 and Flt-1 (Refs. 29, 30; data not shown). We also determined gene expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 because the balances among these proteins are presumed to have crucial roles in the degradation of extracellular matrix in the liver (31, 32). MMP-9 and TIMP-1 gene expression was enhanced in the liver later than 6 h

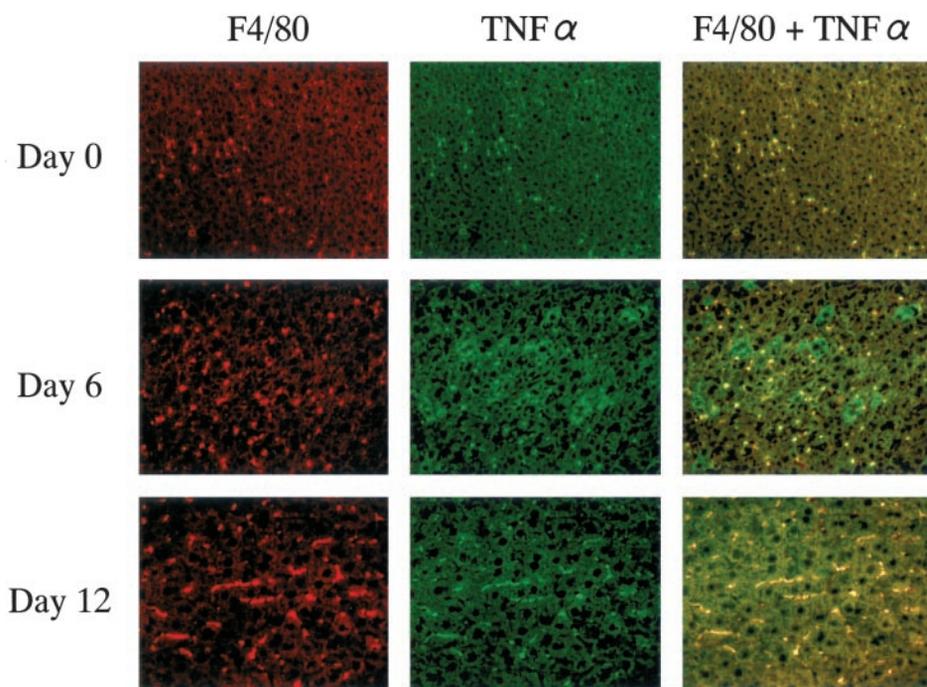


Fig. 3. A double-color immunofluorescence analyses of the liver tissues of WT mice using the combination of anti-F4/80 and anti-TNF- α antibodies. A double-color immunofluorescence was performed on the livers of WT mice at the indicated time intervals after the intrasplenic tumor injection. The figures in the first column and the second column indicate F4/80-positive (red) and TNF- α -positive (green) cells, respectively. The figures in the third column indicate the presence of double-positive cells (yellow). Representative results from three independent experiments are shown. Original magnification, $\times 200$.

Table 2 *Metastasis assay*

Colon 26 cells (5×10^4) were injected into the spleen of WT and TNF-Rp55-deficient mice. Mice were sacrificed at 24 days after tumor injection to determine the incidence of liver metastasis, the numbers of metastatic foci in the liver, and tumor sizes. Tumor sizes were calculated by an equation of $a \times b^2/2$, where a and b indicate long and short diameters of the tumor, respectively. The parameters of liver weight and tumor size were determined in mice, which developed liver metastases. Each value represents mean \pm SE.

| | TNFRp55 KO | WT | P |
|---------------------------|--------------------|---------------------|-------|
| Liver metastasis | | | |
| Incidence | 12/26 (46.2%) | 24/26 (92.3%) | 0.002 |
| Weight (g) | 1.47 \pm 0.19 | 1.70 \pm 0.27 | 0.012 |
| Volume (mm ³) | 147.0 \pm 131.8 | 287.9 \pm 186.4 | 0.026 |
| (Range, median) | (8.0–507.5, 108.0) | (94.5–671.5, 220.5) | |
| Maximum (mm) | 5.0 \pm 1.9 | 7.1 \pm 1.8 | 0.003 |
| Number | 2.9 \pm 1.7 | 4 \pm 1.8 | 0.1 |
| Spleen tumor | | | |
| Diameter (mm) | 14.2 \pm 4.4 | 12.9 \pm 4.9 | 0.31 |
| Weight (g) | 1.11 \pm 0.15 | 1.03 \pm 0.21 | 0.29 |

after the intrasplenic tumor injection, whereas MMP-2 and TIMP-2 gene expression did not change significantly (data not shown). However, there were no significant differences in MMP-9 and TIMP-1 gene expression between WT and TNF-Rp55 KO mice (data not shown). Thus, the reduced incidence of liver metastasis in TNF-Rp55

KO mice cannot be ascribed to changes in angiogenic factor, MMP, and/or TIMP expression.

Effect of Intrasplenic Tumor Injection on VCAM-1 Expression in the Liver of TNF-Rp55 KO Mice. Several lines of evidence indicate that the development of metastasis requires an interaction between tumor and endothelial cells mediated by adhesion molecules including E-selectin, ICAM-1, and VCAM-1 (33–38). Hence, we examined the expression of these adhesion molecules by immunohistochemical analysis. ICAM-1 protein was detected on the sinusoidal endothelial cells of untreated WT and TNF-Rp55 KO mice (data not shown). ICAM-1 expression did not change significantly after tumor or vehicle injection into WT and TNF-Rp55 KO mice (data not shown). Both VCAM-1 and E-selectin were infrequently detected in the livers of untreated WT and TNF-Rp55 KO mice (Fig. 4). However, VCAM-1 and E-selectin expressions were enhanced in the livers of WT mice, particularly on endothelial cells after the intrasplenic tumor injection. There were no significant differences in E-selectin expression between WT and TNF-Rp55 KO mice. However, VCAM-1 expression was not markedly enhanced in the livers of TNF-Rp55 KO mice after the tumor injection when compared with WT mice (Fig. 4). RT-PCR analysis also demonstrated that VCAM-1

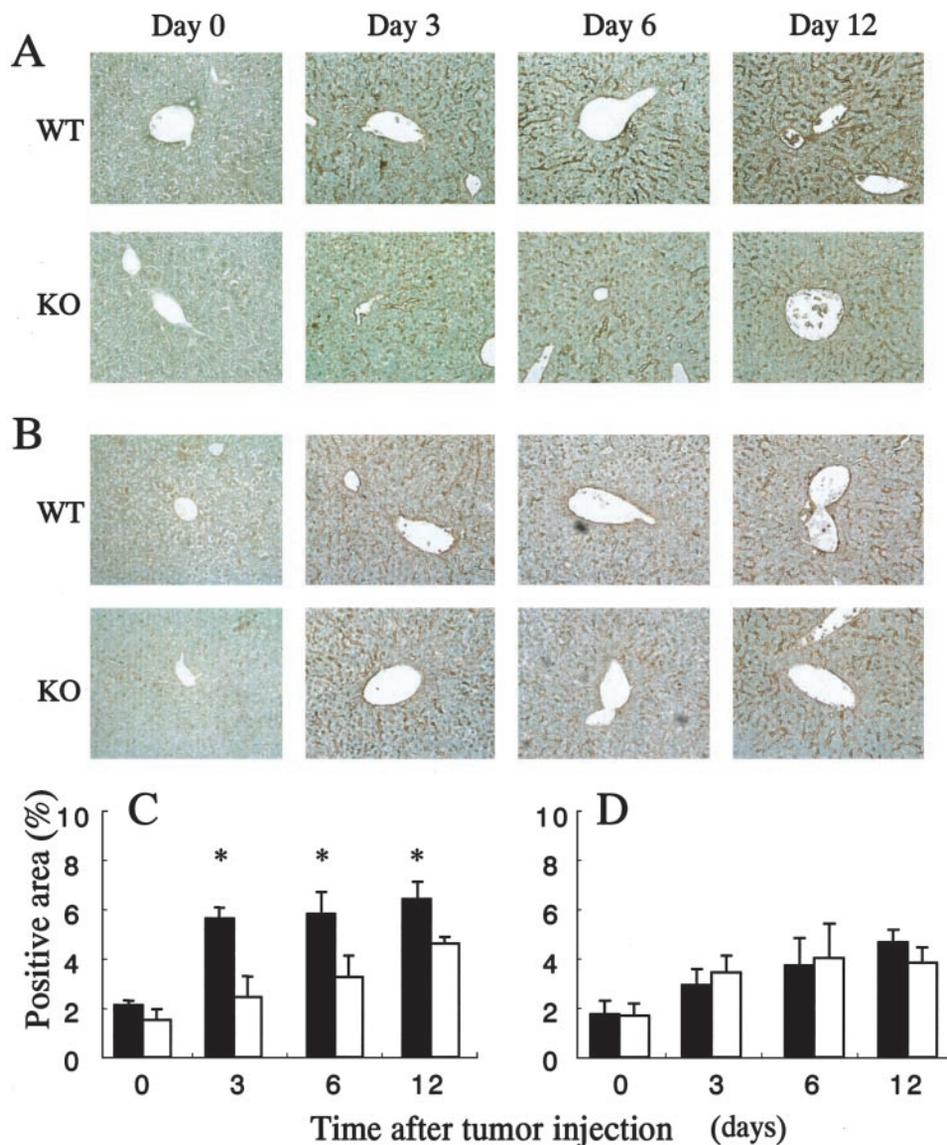


Fig. 4. Immunohistochemical detection of adhesion molecules in the liver. Liver tissues were obtained from WT and TNF-Rp55 KO mice at the indicated time intervals after intrasplenic tumor injection. The sections were immunostained with an anti-VCAM-1 (A) or anti-E-selectin monoclonal antibody (B). Representative results from 3 independent animals are shown in A and B. Original magnification, $\times 200$. VCAM-1- (C) or E-selectin-positive areas (D) of tumor-injected WT mice (■) and TNF-Rp55 KO mice (□) were determined as described in "Materials and Methods." The proportion of positive area were calculated on 6 individual animals and are shown as mean; bars, \pm SE. *, $P < 0.05$.

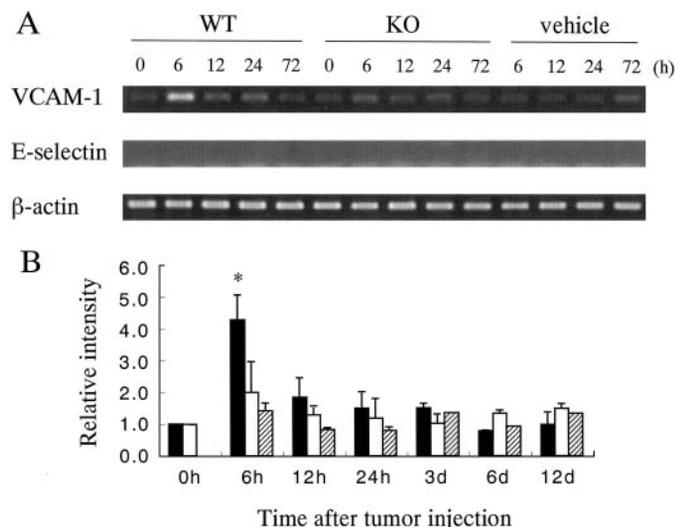


Fig. 5. Adhesion molecule gene expression in the liver. Total RNAs were extracted from the livers of WT and TNF-Rp55 KO mice at the indicated time intervals. RT-PCR analyses were performed and representative results from three individual experiments are shown in A. The ratios of VCAM-1 to β -actin of the tumor-injected WT mice (closed bars), the tumor-injected TNF-Rp55 KO mice (open bars) and the vehicle-injected WT mice (hatched bars) were calculated by designating the ratio of the untreated WT mice as 1.0. Mean \pm SEM. ($n = 6$) are shown in B. *: $P < 0.05$.

gene expression was enhanced in WT but not TNF-Rp55 KO mice (Fig. 5). Moreover, immunofluorescence analysis demonstrated that colon 26 cells expressed a ligand for VCAM-1 and VLA-4 (integrin $\alpha 4/\beta 1$; data not shown). Collectively, these results suggest that TNF-Rp55-mediated signals selectively up-regulate intrahepatic VCAM-1 expression and promote subsequent liver metastasis.

Effect of Intrasplenic Tumor Injection on TNF- α Expression in TNF-Rp55 KO Mice. Because TNF- α can augment its own mRNA expression and protein production (39), we examined TNF- α mRNA expression in the liver after intrasplenic tumor injection. TNF- α mRNA expression was enhanced significantly in the livers of WT but not TNF-Rp55 KO mice, 6 h after the tumor injection (Fig. 1). Immunohistochemical analysis also demonstrated enhanced TNF- α protein expression in WT but not TNF-Rp55 KO mice (data not shown). In contrast, IL-1 α and IL-1 β mRNA expression was enhanced to a similar extent in both WT and TNF-Rp55 KO mice later than 6 h after intrasplenic tumor injection (Fig. 1). These results suggest that the absence of TNF-Rp55 abolished an autocrine amplifying loop between TNF- α and TNF-Rp55, eventually resulting in selectively reducing TNF- α production.

DISCUSSION

TNF- α exhibits cytotoxic and/or cytostatic activities against a wide variety of tumor cells *in vitro* (5), and the administration of TNF- α was effective against various types of tumor models, including those undergoing metastasis (6, 7). Several clinical trials have also demonstrated TNF- α to be effective against several types of advanced metastasizing cancers (12, 13). On the other hand, several lines of evidence suggest that TNF- α can augment the capacity of some tumor cells to metastasize (14–17). Thus, the effects of exogenously administered TNF- α and the endogenously produced TNF- α on metastasis are difficult to predict. Therefore, we compared the severity of liver metastasis in WT and TNF-Rp55 KO mice. This revealed that the incidence of liver metastasis was significantly reduced in TNF-Rp55 KO mice compared with WT mice, implying that TNF-Rp55-mediated signals have important roles in the process of liver metastasis.

In the first step of metastatic invasion, tumor cells interact with

endothelial cells through the use of several distinct adhesion molecules. Several lines of evidence indicate that TNF- α can up-regulate the endothelial expression of E-selectin, which mediates interactions of tumor cells with the endothelium cells (33). Moreover, metastatic spread to the liver of a Lewis lung carcinoma subclone was inhibited by an anti-E-selectin antibody (34), implicating E-selectin as a crucial adhesion molecule in tumor metastasis. However, under our experimental conditions, we did not see any differences in E-selectin mRNA and protein expressions between WT and TNF-Rp55 KO mice at any time points after intrasplenic tumor injection. However, VCAM-1 gene and protein expressions were markedly enhanced in WT but not TNF-Rp55 KO mice. VCAM-1 mRNA expression can be transiently up-regulated by TNF- α (35, 40). VCAM-1 mRNA expression can lead to persistent VCAM-1 protein expression on the cell surface (40), which mediates the interaction of the tumor cells with endothelial cells *in vitro* (36). Moreover, immunofluorescence analysis demonstrated that colon 26 cells expressed VLA-4 on the surface. VLA-4, usually expressed on leukocytes (41), is also found on malignant melanoma (42, 43) and renal cell carcinoma (44). VLA-4 expressed by tumor cells is associated with the augmentation of experimental metastasis induced by IL-1 or TNF- α (42, 43). Thus, it is likely that interaction of VCAM-1 with VLA-4 is also involved in this metastasis process.

Consistent with our observations, Khatib *et al.* (37) reported that both IL-1 and TNF- α mRNA expressions were very rapidly augmented in the liver within 6 h after intrasplenic tumor injection. We additionally demonstrated that TNF- α protein expression was enhanced around central and portal veins, which are presumed to be the entry site of tumor cells to the liver. Thus, rapidly migrating tumor cells may induce the expressions of IL-1 and TNF- α . TNF- α , in synergy with IL-1, rapidly enhances the expression of VCAM-1, thereby increasing the interaction between tumor and endothelium cells, the first step of tumor colonization of an organ. Subsequent steps require the matrix degradation by matrix proteinases and angiogenesis. However, TNF-Rp55-mediated signals have few, if any, roles in these latter steps, at least in this model, based on our observation that intrasplenic tumor injection caused no significant differences in angiogenic factor, MMP, and TIMP gene expression between WT and TNF-Rp55 KO mice. Additional studies may be required to ascertain whether TNF-Rp55-mediated signals have similar effects in other mouse metastasis models and human cancer. If so, the initial stage of tumor metastasis may effectively be prevented by the administration of TNF antagonists, which are now in clinical trials for nonmalignant diseases such as rheumatoid arthritis (45) and inflammatory bowel diseases (46).

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