Jpn. J. Cancer Res. 84, 1150–1158, November 1993

Immunohistochemical Detection of Hepatocyte Growth Factor/Scatter Factor in Human Cancerous and Inflammatory Lesions of Various Organs

Yasuteru Yoshinaga,[1,2] Yoshihiro Matsuno,[1] Shin Fujita,[1] Toshikazu Nakamura,[3] Masahiro Kikuchi,[2] Yukio Shimosato[4] and Setsuo Hirohashi[1,5]

[1]*Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104,* [2]*First Department of Pathology, School of Medicine Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-01,* [3]*Department of Biology, Faculty of Science, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812 and* [4]*Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104*

Hepatocyte growth factor (HGF)/scatter factor (SF) is a multifunctional factor considered to be potentially involved in tissue regeneration, wound healing, embryogenesis, angiogenesis and cancer invasion. Here we examined immunohistochemically the distribution of HGF/SF in human tissues, including cancerous and inflammatory tissues, using anti-HGF antibody. HGF/SF accumulation was clearly detected in the extracellular matrix, particularly along the basement membrane, in cancerous and inflammatory tissues, but only a little was detected in normal tissues. HGF/SF is well known to have a strong affinity for heparin *in vitro*, and from the results of our immunohistochemical assay, we considered that HGF/SF was bound to heparin or heparan sulfate of the extracellular matrix and basement membrane. HGF/SF was well localized in cancerous and inflammatory lesions of human lung, liver and pancreas, and in apparently normal tissues of kidney, adrenal gland and pancreas obtained at autopsy. In lung, HGF/SF was localized along the basement membranes of cancer cell nests, in the extracellular matrix of the cancer cell surface, cancer stroma and tissues invaded by cancer, and the basement membranes of bronchial epithelium and capillary vessels in inflammatory stroma. Since HGF/SF makes some cancer cells more invasive *in vitro*, the accumulation of HGF/SF in cancerous tissue suggests that the invasiveness of some cancer cells may be increased by HGF/SF *in vivo*.

Key words: Immunohistochemistry — Hepatocyte growth factor — Extracellular matrix — Basement membrane — Cancer invasion

Hepatocyte growth factor (HGF) is thought to be of major importance for liver regeneration.[1,2] In fact, HGF is a potent mitogen for hepatocytes in primary culture. Rat HGF was first identified in serum following partial hepatectomy,[3] and subsequently it was purified to homogeneity from platelets[4] and damaged liver.[5] In several laboratories, human HGF has also been isolated from serum of patients following partial hepatectomy,[6] plasma of patients with fulminant hepatitis[7] and liver cirrhosis,[8] ascites of patients with liver cirrhosis,[8] and also from placenta[9] and the conditioned medium of human embryonic lung fibroblasts.[10] cDNA of human HGF has been cloned and the coded amino acid sequence determined.[10–12] HGF mRNA is known to be expressed in several tissues of rat[13] and human,[14] such as lung, liver, kidney, brain and thymus. Zarnegar *et al.*[15] and Wolf *et al.*[9] studied the tissue distribution of HGF by immunohistochemistry in several organs of normal rabbit, and in human placenta and trophoblastic disease, respectively. HGF[5] and its mRNA[13,14,16] are markedly increased in rat liver after injury by hepatotoxins or hepatectomy. Human HGF is also increased significantly in the serum of patients with various liver diseases.[17] On the other hand, HGF is not a specific growth factor for hepatocytes because it also markedly stimulates DNA synthesis by renal tubular epithelial cells, keratinocytes and melanocytes.[18,19] Moreover, the HGF gene is expressed in the lung and kidney in cases of hepatitis.[20] These results indicate that HGF may be a ubiquitous factor whose function is mediated in a paracrine or endocrine manner, stimulating the growth of various epithelial cells; an increase of HGF seems to be closely related to tissue damage or certain diseases.[1,2]

Scatter factor (SF), on the other hand, is a cytokine released by human embryonic fibroblast cell lines and variant or transformed derivatives of the 3T3 cell line, and has potency to dissociate certain epithelial cells from each other *in vitro*.[21] Besides the conditioned medium of fibroblast cell lines,[22,23] SF-like activity has also been isolated from human placenta[24] and the conditioned medium of human vascular smooth muscle cells.[25] SF affects the motility, morphology, and mutual attachment of certain epithelial cells and vascular endothelial cells in culture, but it exerts no effect on fibroblasts.[21,26] Accord-

[5] To whom reprint requests should be addressed.

ingly, the functions of SF are considered to be modulated in a paracrine manner. Furthermore, some cancer cell lines show sensitivity to SF,[23,25] and in fact SF increases the invasiveness of cancer cells in an invasion assay system *in vitro*.[23] From all of these reported results, it is reasonable to consider that SF might be involved in processes such as embryonic development, wound healing, vascular repair and cancer invasion.

Recent studies have revealed that HGF and SF are identical proteins with regard to biological activity, immunological characteristics and amino acid sequence.[23,27,28] HGF/SF is a heterodimeric molecule composed of large (Mr 57–69 kDa) and small (Mr 30–34 kDa) subunits, and the primary structure of each subunit has been determined.[11,13] HGF/SF is now thought to be a multifunctional factor that plays important roles *in vivo*. However, its distribution in human tissues has not been fully established. To understand the role of HGF/SF *in vivo*, it is necessary to investigate its distribution in human tissues, particularly in cancerous and inflammatory lesions. In the present study we addressed this issue by using immunohistochemistry with an anti-HGF polyclonal antibody.

MATERIALS AND METHODS

Antibody A polyclonal antibody raised in rabbit against recombinant human HGF[29] was purified using a Protein A-Sepharose column.[27] In Western blot analysis, this antibody specifically detected only the 82-kDa HGF protein under non-reducing conditions in the conditioned medium of MRC5, a human embryonic fibroblast cell line obtained from the Japanese Cancer Research Resource Bank (Tokyo) (data not shown).

Immunohistochemistry Most human tissues were obtained at surgery at the National Cancer Center Hospital. Non-cancerous tissues obtained from six autopsy cases were also examined. Metastatic cancers in lung and liver were from colorectal adenocarcinomas. The tissues were processed by the AMeX method.[30] Briefly, the tissues were fixed in acetone at −20°C overnight, dehydrated in acetone at 4°C for 15 min, and then at room temperature for 15 min, cleared in methyl benzoate for 30 min and then in xylene for 30 min consecutively, and finally penetrated with paraffin wax. Sections 2 μm thick were cut and deparaffinized with xylene, immersed in acetone and incubated in methanol with 0.3% hydrogen peroxide to block endogenous peroxidase activity for 30 min at room temperature. After rehydration with phosphate-buffered saline (PBS), the sections were preincubated with 2% normal swine serum (NSS) in PBS, and then incubated with either polyclonal antibody against recombinant human HGF or control non-immunized rabbit serum diluted 1:2000 with 2% NSS at room temperature for 2 h. The sections were washed three times in PBS, and incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 with 2% NSS for 30 min. After washing in PBS three times, they were incubated with avidin-biotinyl peroxidase complex prepared using a Vectastain ABC kit (Vector) for 30 min. They were washed with PBS, and then incubated in a solution containing 0.02% diaminobenzidine and 0.03% hydrogen peroxide in 0.1 *M* Tris-HCl buffer, pH 7.6, for 5 min. Nuclear counterstaining was performed with Mayer's hematoxylin solution.

Immunohistochemical assay of HGF/SF binding to tissue After deparaffinization and incubation with methanol, the AMeX tissue sections were incubated with or without heparinase-I (0.5 unit/ml in PBS, Sigma, St. Louis, MO) at 37°C for 30 min. They were washed in PBS and incubated with recombinant HGF (0.1 μg/ml) at 4°C overnight. After washing in PBS, HGF/SF was visualized with the polyclonal antibody as described above.

RESULTS

Distribution of HGF/SF in non-cancerous tissues We stained non-cancerous tissues, located sufficiently distant from areas of cancer, in several organs obtained at surgery and autopsy.

HGF/SF was localized along the basement membrane, thickened stroma, and vessel walls in inflammatory tissues (Table IA), being stained particularly clearly and strongly along the basement membrane. HGF/SF was not detected in many tissues lacking marked inflam-

Table I. Distribution of HGF/SF in Non-cancerous Tissues

(A) Inflammatory tissues

Tissue	No. of cases	No. showing positive staining
Lung	41	36
Liver	7	7
Kidney	1	1

(B) Non-inflammatory tissues

Tissue	No. of cases	No. showing positive staining
Lung	44	3
Liver	7	1
Kidney	4	2
Adrenal gl.	4	3
Pancreas	5	3
Spleen	5	0
Stomach	8	0
Colon	3	0
Thyroid	6	0
Testis	4	1

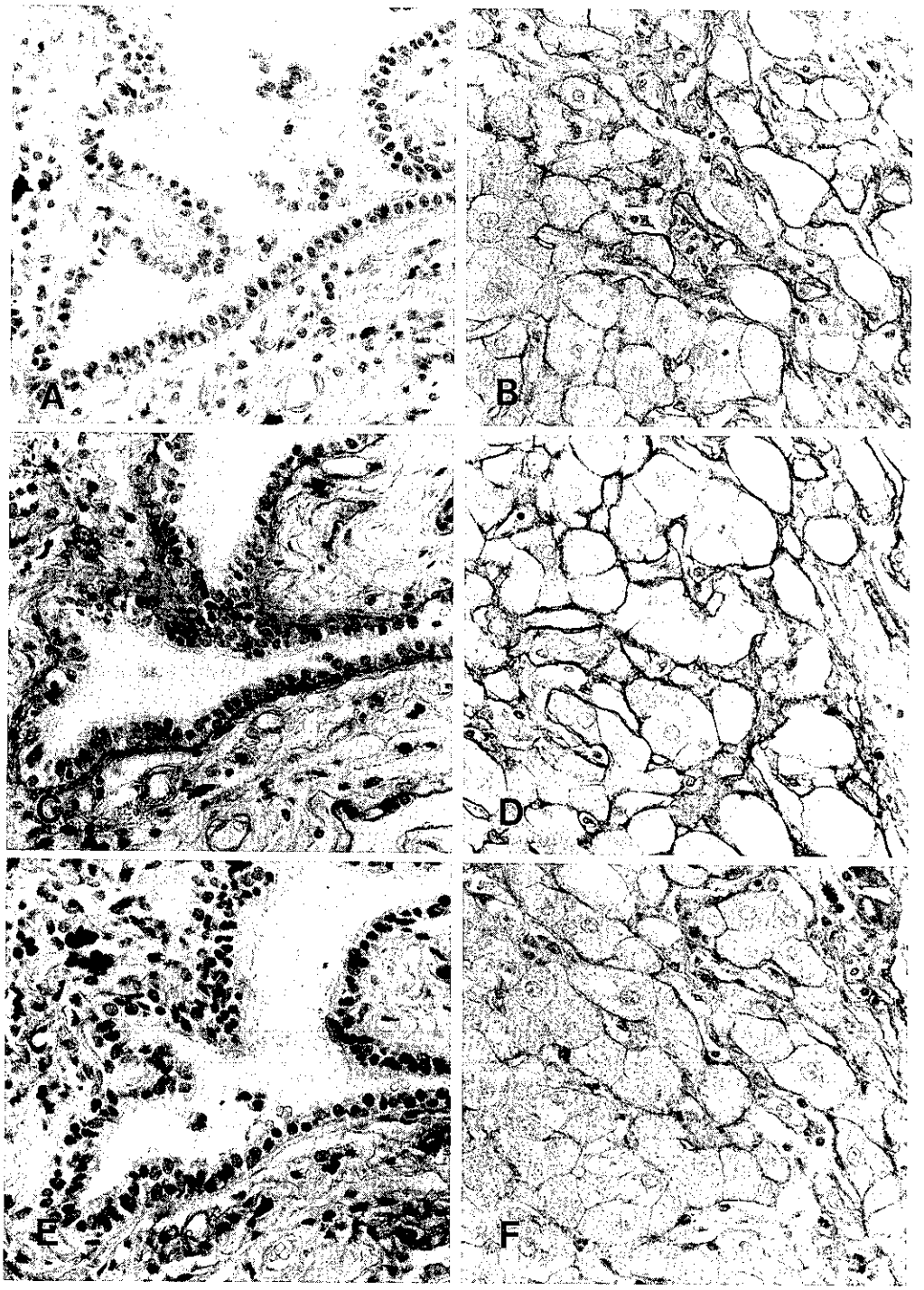

Fig. 1. Normal lung, liver cirrhosis and immunohistochemical assay of HGF/SF binding to tissue. A and B: Distribution of endogenous HGF/SF; (A) Normal lung; no positive staining. (B) Liver cirrhosis; deposition of HGF/SF is evident along the basement membrane of the sinusoidal capillaries and cell surface of hepatocytes. C and D: After incubation with recombinant HGF/SF; positive staining is present along the basement membrane of the bronchial epithelium (C) and positive staining is enhanced (D). E and F: After incubation with recombinant HGF/SF following heparinase treatment; note disappearance of positive staining of exogenous HGF/SF. A, C, E and B, D, F are serial sections of the same tissues, respectively. Original magnification, ×400.

matory changes (Fig. 1A), but was detected clearly in apparently normal tissues of kidney, adrenal gland and pancreas obtained at autopsy (Table I).

In the lung, HGF/SF was stained at the basement membrane of bronchioli, alveoli and proliferating capillary vessels in inflammatory stroma, and broad alveolar septa in cases of interstitial pneumonia, obstructive pneumonia and bullous emphysema (Fig. 2A).

In cirrhotic or precirrhotic liver, HGF/SF was stained in the following areas: i) basement membrane of sinusoidal capillaries, vessels and pseudobile ducts in inter-pseudolobular septa, ii) inter-pseudolobular septal stroma, iii) cell surface of hepatocytes (Fig. 1B and 2B). The staining of the basement membrane of sinusoidal capillaries was stronger in areas close to inter-pseudolobular septa than in the central area of pseudolobules.

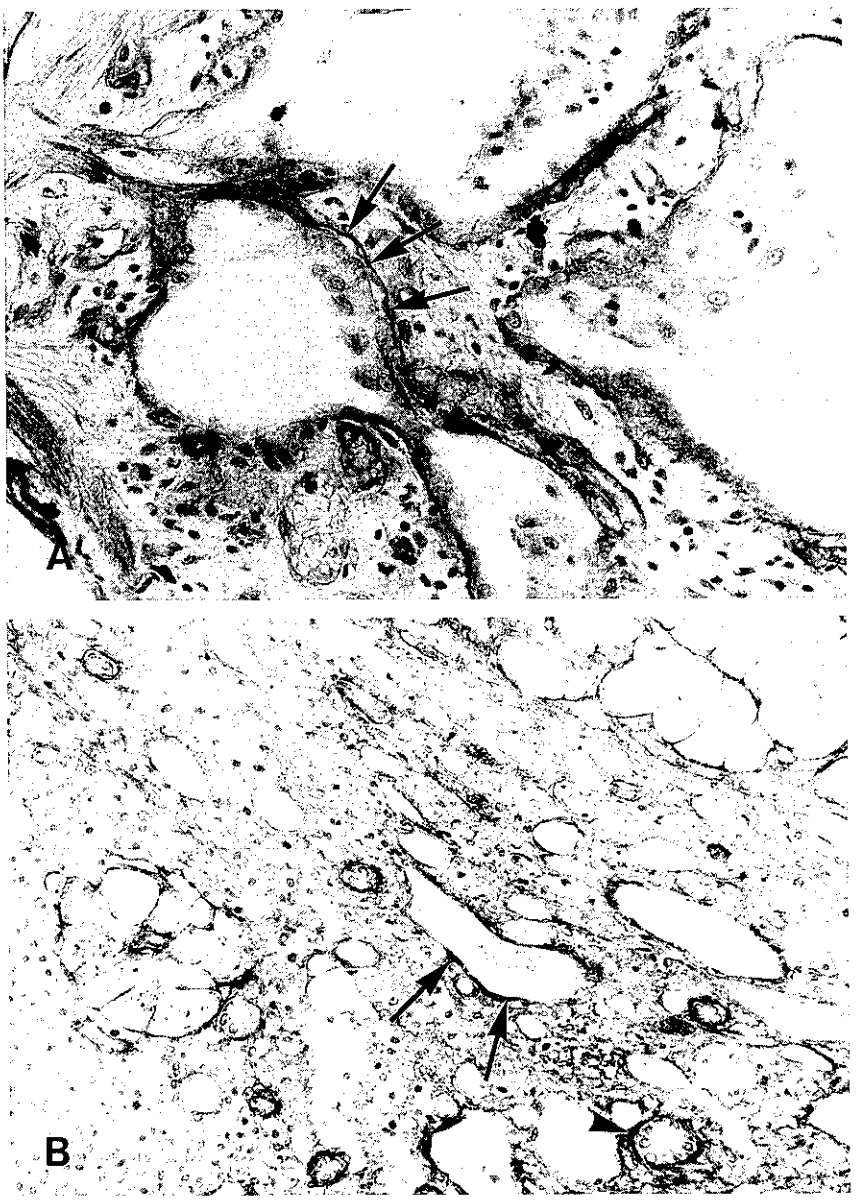

Fig. 2. Inflammatory lesions in lung and liver. A: Inflammatory change in lung; the basement membrane of the alveolar epithelium is positively stained. B: Interpseudolobular septa of liver cirrhosis; intense staining is evident along the basement membrane of capillary vessels (arrow) and pseudobile ducts (arrowhead), and positive staining is present in the septal stroma. Original magnification, ×400.

When there were no inflammatory changes such as cirrhosis and chronic hepatitis, no such staining was detected.

In kidney, HGF/SF was stained along the basement membrane of urinary tubules and Bowman's capsule.

In pancreas, deposition of HGF/SF was observed along the basement membrane of the pancreatic duct, ductules and exocrine acini, in addition to vessel walls. Islet cells themselves did not show any positive reaction, in contrast to the basement membrane of the capillary vessels within each islet.

Distribution of HGF/SF in cancerous lesions In cancerous lesions of the lung, liver, pancreas and biliary tract, HGF/SF showed strong staining with the same pattern as that in inflammatory lesions without cancer. However, in the tissue of stomach cancer, only a little HGF/SF was detected (Table II).

In lung cancer, HGF/SF was stained along the basement membrane of cancer cell nests (Fig. 3B), and extracellular matrix of the cancer cell surface and cancer stroma. Moreover, positive staining was seen along the thick basement membrane of bronchial epithelium and capillaries in the tissue surrounding the cancer (Fig. 3A), in a manner very similar to that in non-cancerous inflammatory lesions. In poorly differentiated carcinoma where the basement membrane was almost lost, HGF/SF was stained mainly in the broad fibrotic stroma (Fig. 3C). Fibroblasts in the cancer stroma seemed to show positive staining, although the staining pattern was not well defined (Fig. 3C).

In hepatocellular carcinoma, most of which was associated with liver cirrhosis, in addition to the positive staining in areas of liver cirrhosis, HGF/SF was stained along the basement membrane of sinusoidal capillaries of cancer cell nests and on the surface of cancer cells near the cancer stroma (Fig. 4). By contrast, in metastatic liver cancers, HGF/SF was detected in the stroma and basement membrane of sinusoidal capillaries around the cancer mass, but only weakly within the cancer mass.

Table II. Distribution of HGF/SF in Cancerous Tissues

Tissue	No. of cases	No. showing positive staining
Lung cancer		
primary	85	76
metastatic	3	3
Liver cancer		
primary	8	6
metastatic	3	3
Breast cancer	8	2
Pancreas & biliary tract cancer	14	11
Stomach cancer	6	1

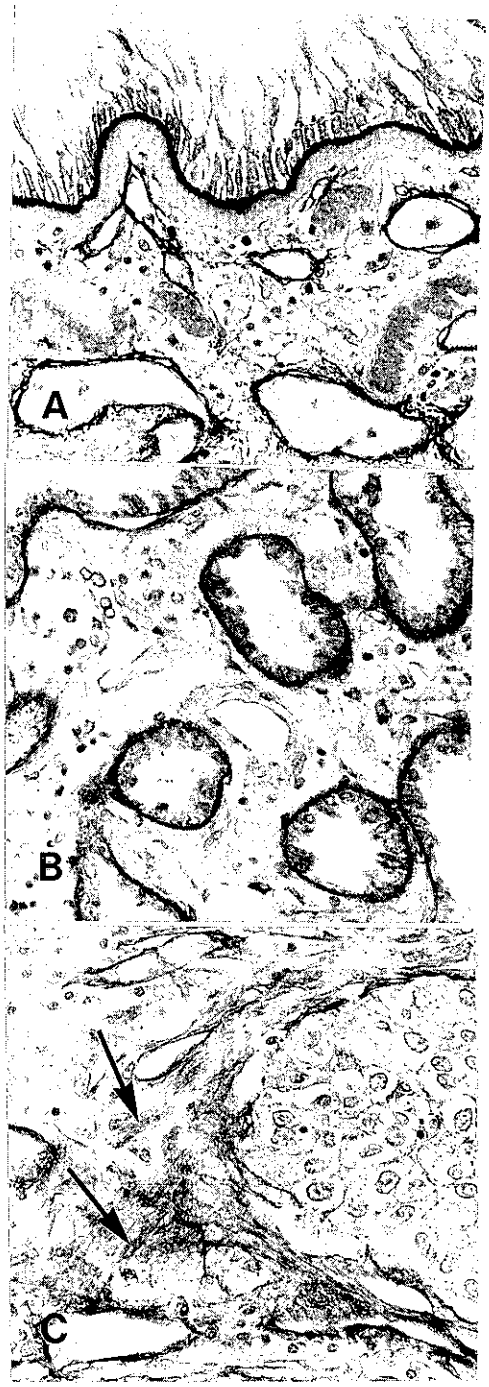

Fig. 3. Cancerous lesions in lung. A: Bronchial epithelium in the vicinity of cancer; there is prominent staining of the basement membrane of the bronchial epithelium and small vessels in the inflammatory stroma. B: Well differentiated adenocarcinoma; the basement membrane of cancer nests is positively stained. C: Poorly differentiated carcinoma; there is intense staining of the stroma surrounding the cancer and faint surface staining of cancer cells and fibroblasts (arrow). Original magnification, ×400.

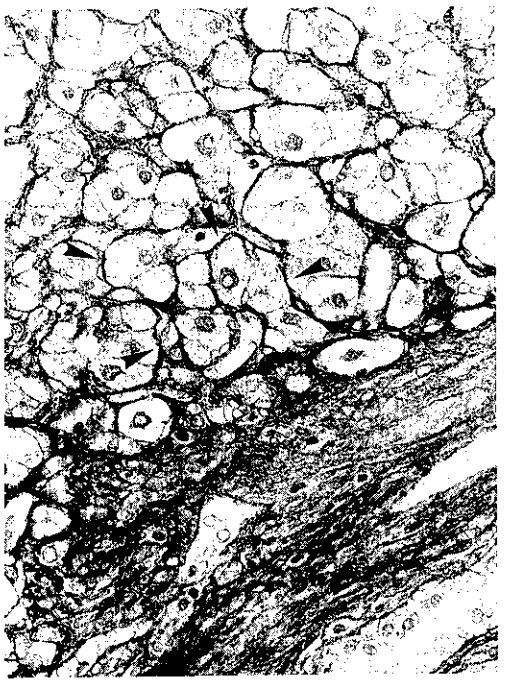

Fig. 4. Lesion of hepatocellular carcinoma: positive staining is evident along the basement membrane of sinusoidal capillaries facing cancer cells and cancer cell surface, near the intensely stained cancer stroma. Original magnification, ×400.

HGF/SF was not clearly detected in the cytoplasm of endothelial cells, macrophages or cancer cells.

Binding of HGF/SF to heparin moieties in tissues The tissue distribution of exogenous HGF/SF after incubation of tissue with the factor was exactly the same, but much stronger, than that of endogenous HGF/SF (Fig. 1C and 1D), and this binding of exogenous HGF/SF to tissue was completely abolished after incubation of the tissue with heparinase (Fig. 1E and 1F). These results indicate that HGF/SF is bound to heparin or heparan sulfate in the tissue extracellular matrix.

DISCUSSION

In this study we have demonstrated that HGF/SF is clearly localized in the extracellular matrix, especially the basement membrane, whose major constituents are heparan sulfate and glycosaminoglycan related to heparin, or on the surface of cancer cells and hepatocytes, due to binding to heparin or heparan sulfate. In fact, the strong affinity of HGF/SF for heparin has been utilized in several laboratories for purification of HGF/SF using a heparin-affinity column. Recent studies have shown that target cells, hepatocytes or some cancer cells, have two binding sites for HGF/SF.[31, 32] It is suggested that one binding site with relatively low affinity is due to heparan sulfate proteoglycans, whereas the other with high affinity is the specific receptor of HGF/SF. Our results indicate that at least the low-affinity binding of HGF/SF is detectable immunohistochemically in human tissues with anti-HGF antibody, although the high-affinity binding of HGF/SF, and HGF/SF in its producer cells, is not well defined. One explanation for this may be that there is much less HGF/SF at high-affinity binding sites than at low-affinity ones. Rapid turnover of HGF/SF in its producer cells and on the specific receptors is another possibility. Previous immunohistochemical studies of HGF/SF in normal rabbit[15] and human placenta and trophoblastic disease[9] revealed that HGF/SF was expressed in the cytoplasm of several tissues, but we were unable to detect HGF/SF in cytoplasm of any cells other than fibroblasts, which seemed to be positively stained in some cases. We considered that this difference was due to the different anti-HGF/SF antibodies used and the method of fixation. Our results regarding the tissue and organ localization of HGF/SF *in vivo* were well consistent with the expression of HGF mRNA in normal adult rats.[13]

Fibroblast growth factors (FGFs), which, like HGF/SF, are heparin-binding proteins, are reported to be present in the extracellular matrix,[33] and basic fibroblast growth factor (bFGF) in particular has been detected in the basement membrane of bovine cornea by immunofluorescence.[34] It is interesting that the localization of bFGF, which is strongly expressed immunohistochemically in tissue with chronic inflammation,[35] is similar to that of HGF/SF. Therefore, HGF/SF and bFGF might show the same kinetics, tissue localization and movement, *in vivo*. The binding of HGF/SF or FGFs to heparin or heparan sulfate has been considered to protect the factors from degradation, or to store them until required for binding to their specific receptor.[36, 37] HGF/SF bound to the extracellular matrix, including the basement membrane, might be released and gain biological activity, like FGFs,[33, 34] when the extracellular matrix bearing HGF/SF is exposed to heparinase-like enzyme (e.g. that produced by cancer cells[38]) and/or when it is directly destroyed by cancer cells. Subsequently, local motility or proliferation of epithelial cells, cancer cells and endothelial cells may increase.

Because HGF/SF is a systemic circulating factor in patients with fulminant hepatitis[7] or liver cirrhosis,[8] it is reasonable to consider that it also circulates in the body of cancer patients, who usually suffer from severe inflammation. Therefore, the tissues without cancer or inflammation which we obtained were not completely "normal," since many were from patients bearing cancer and concomitant inflammation elsewhere in the body. Because specimens of lung, kidney, pancreas and adrenal

gland obtained at autopsy tend to have many inflammatory and postmortem changes, we cannot exclude the possibility that positive staining in tissue sections of such "normal" organs might reflect the presence of some disorders in the sampled area. However, our results show that HGF/SF was stained little or only very weakly along the basement membrane of many "normal" tissues which were located distantly from cancer and inflammation, and the distribution of HGF/SF clearly differed between human cancerous or inflammatory lesions and "normal" tissues. As to the reason for this difference, we offer the following two hypotheses: i) HGF/SF may bind to extracellular matrix easily when the latter is exposed to HGF/SF in cases of tissue destruction due to wounding, cancer invasion and inflammation. Because the basement membrane of the liver cell plate is always exposed to blood, the membrane may become bound easily to heparin-binding protein. ii) Large amounts of HGF/SF may be produced in cancerous and inflammatory lesions, especially those of the lung and liver. Noji *et al.* showed that expression of HGF/SF in rat liver after injection of hepatotoxin was confined to endothelial and Kupffer cells, by *in situ* hybridization.[20] We were unable to define the cells producing HGF/SF by immunohistochemistry, but fibroblasts of cancer stroma seemed to be stained in some cases. As HGF/SF is produced by human embryonic fibroblasts,[21] it is probable that HGF/SF is also produced by activated fibroblasts in cancerous or inflammatory tissues. In fact, we confirmed by scattering bioassay and Western blot analysis that primary-cultured lung fibroblasts from lung cancer patients had ability to release HGF/SF into the culture medium (data not shown).

Furthermore, our data indicate that HGF/SF is not localized in all tissues and organs bearing cancer or inflammation, but seems to be localized mainly in lung, liver, kidney, adrenal gland and pancreas, as far as we examined. Tashiro *et al.* have shown that HGF mRNA is expressed in lung, liver, kidney, brain and thymus of normal adult rats,[13] and Selden *et al.* have demonstrated that human HGF mRNA is strongly expressed in human fetal liver and in adult liver following hepatectomy.[14] Although our results are consistent with these data, it is necessary to conduct analysis at the mRNA level in cancerous and inflammatory tissues in order to identify the precise sites and cells producing human HGF/SF *in vivo*.

HGF/SF exists in human cancerous tissue. Interestingly, some cancer cells have sensitivity to HGF/SF, and their invasive potential is increased in the presence of HGF/SF *in vitro*.[23] We have also confirmed this phenomenon in the T24 human bladder cancer cell line using a modified Boyden chamber with Matrigel (data not shown). We suggest that HGF/SF is induced *in vivo* specifically for tissue repair, tissue regeneration and angiogenesis in cooperation with other factors (e.g. FGFs) as a reaction to tissue damage including inflammation or cancer, and that consequently the motility and invasiveness of some cancer cells may be increased.

Recently the c-*met* proto-oncogene product was identified as the receptor of HGF/SF.[32, 39, 40] It will be necessary to investigate the *in vivo* distribution of c-*met* and its product in comparison with HGF/SF to clarify the physiological role of HGF/SF, especially under conditions of cancer invasion.

ACKNOWLEDGMENTS

We thank Mr. T. Osaka for photographic work.

(Received May 18, 1993/Accepted August 9, 1993)

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