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Appearance of Osteonectin-expressing Fibroblastic Cells in Early Rat Stomach Carcinogenesis and Stomach Tumors Induced with N-Methyl-N′-nitro-N-nitrosoguanidine

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The present study was designed to define molecular alterations in the initiation stage of rat stomach carcinogenesis. Groups of male Lewis rats, 6 weeks old, were given drinking water with or without N-methyl-N′-nitro-N-nitrosoguanidine (MNNG; 100 mg/liter). Total RNA was isolated from the stomach pyloric mucosa, and fluorescent differential display analysis was performed. A cDNA fragment of 125 bp encoding an extracellular matrix-associated matricellular glycoprotein, osteonectin, was identified after 14 days of MNNG exposure. A severalfold increase in expression was observed after 14 and 27 days of MNNG exposure, as determined by northern blot and RT-PCR. Immunohistochemistry revealed that osteonectin-mAb-stained fibroblastic cells appeared in interstitial tissue of pyloric mucosa. Additionally the gene expression of other extracellular matrix proteins, viz., collagen type III, fibronectin, osteopontin, proteoglycan NG2, laminin γ1 and S-laminin, was also markedly increased, as determined by competitive RT-PCR after 14 days of MNNG exposure. The gene expression of osteonectin and the six other extracellular matrix proteins was elevated in twelve stomach adenocarcinomas and adenomas induced by MNNG in Lewis and WKY rats. Osteonectin-mAb-stained fibroblastic cells were evident in interstitial tissue of stomach tumor. These results suggest that osteonectin-expressing fibroblastic cells appear in the interstitial tissue of pyloric mucosa from the early initiation stage of rat stomach chemical carcinogenesis, and that this phenomenon probably plays a role in cancer development.

Key words: Osteonectin — Extracellular matrix protein — Rat stomach carcinogenesis — MNNG — Fluorescent differential display

Stomach cancer remains a major cause of cancer mortality in the world.[1)] Several factors such as diet, exogenous chemicals, intragastric synthesis of carcinogens, infection, pathological conditions in the stomach and genetic factors are suspected to play a role in stomach carcinogenesis.[2)] However, the molecular mechanisms of development of stomach cancer are largely undefined. Experimental stomach carcinogenesis is a useful tool to elucidate the molecular mechanisms. Carcinogenesis is thought to be a complex process involving many steps, including at least initiation, promotion and progression. The initiation step differs in different cancers. Identifying genes whose alterations lead to tumorigenesis or carcinogenesis is useful for investigation of molecular mechanisms, cancer prevention, diagnosis and therapy. The aims of the present study are to investigate alterations of gene expression in the initiation stage of stomach carcinogenesis by fluorescent differential display analysis (FDD)[3)] and to evaluate the significance of such alterations.

We have studied various alterations in the initiation stage of experimental stomach carcinogenesis.[4,5)] Previously we found changes in gene expression in the initiation stage of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced rat stomach carcinogenesis by using FDD and we cloned MHC class II-associated invariant chain (Ii).[6)] Then, we found that Ii was expressed in antigen-presenting dendritic cells, and we suggested the involvement of dendritic cell response in resistance to stomach carcinogenesis in a particular rat strain.[7)]

The present study describes the application of FDD to MNNG-induced rat stomach carcinogenesis and the cloning of the *osteonectin* gene, an extracellular matrix-associated matricellular glycoprotein gene. Osteonectin, also called BM40 or SPARC (secreted protein, acidic and rich in cysteine), is a nonstructural component of the extra-

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cellular matrix and is a multifunctional glycoprotein involved in many biological processes, such as tissue mineralization, cell-extracellular matrix interaction and angiogenesis. Osteonectin is associated with cell populations exhibiting high rates of turnover and remodeling.[8, 9] Others have reported changes in osteonectin expression, overexpression and down-regulation in different malignant tumors, including human prostate cancer,[10] breast cancer,[11] colorectal cancer [12] and epithelial ovarian cancer.[13] However, little is known about osteonectin in stomach cancer.[14] It has been suggested that osteonectin may play a key role in tumor invasion and metastasis in certain malignancies.

Additionally, we have studied the expression of osteonectin and, for comparison, that of six other extracellular matrix genes in the initiation stage of stomach carcinogenesis. In order to know whether changes in expression represent a temporary phenomenon only in the early stage of carcinogenesis, expression of osteonectin and these other extracellular matrix genes was studied in rat stomach adenocarcinomas and adenomas induced by MNNG.

MATERIALS AND METHODS

Animals For gene expression and cell proliferation studies in stomach pyloric mucosa, groups of male 6-week-old Lewis rats (LEW/Crj; Charles River Japan, Inc., Yokohama) were used as follows: group 1, ten rats were given MNNG (100 mg/liter, the same concentration as used for long-term stomach carcinogenesis studies; Nacalai Tesque, Inc., Kyoto) in drinking water for 14 days; group 2, ten control rats were given distilled water instead of MNNG; groups 3 and 4, five rats were given MNNG for 3 and 27 days, respectively; group 5, five rats were given MNNG for 27 days and water for 7 days; groups 6–8, five rats were given water for 3, 27, and 34 days, respectively; group 9, five rats were given 10% NaCl diet and water for 14 days. Rats except group 9 had free access to pellet rodent chow (CE-2, Nihon Clea, Tokyo). Rats were killed by cervical dislocation under ether anesthesia. Stomachs from five rats in each group were surgically removed. The pyloric mucosa was scraped off with a blade, pooled, frozen in liquid nitrogen, and stored at −80°C until use. For groups 1 and 2, the other five rats were injected with 5-bromo-2-deoxyuridine (BrdU, 100 mg/kg body weight) intraperitoneally 1 h before killing. Stomachs were washed with cold phosphate-buffered saline (PBS), and fixed in 100% ethanol or frozen in liquid nitrogen for histochemical examination.

Stomach tumors Ten male 6-week-old Lewis and WKY (WKY/NCrj; Charles River Japan) rats were housed in plastic cages on hard wood chip bedding in an air-conditioned room with a 12 h light/dark cycle and were given MNNG (100 mg/liter) in drinking water for 30 weeks. Ten control rats were given tap water without MNNG. Rats had free access to pellet rodent chow (Oriental MF; Oriental Yeast Co., Ltd., Tokyo). Rats were dissected after 50 weeks and stomach tumors were removed and stored at −80°C until use. Portions of the stomach tumors were fixed in 100% ethanol or frozen in liquid nitrogen for histochemical examination. Induced tumors were mostly well-differentiated adenocarcinomas and a few adenomas.

RNA isolation Total RNAs from stomach pyloric mucosa were isolated by a modified method using acid guanine thiocyanate/phenol chloroform using TRIzol (Life Technologies, Inc., Gaithersburg, MD) and ISOGEN LS (Nippon Gene, Toyama) reagents and stored at −80°C until use as described previously.[6] Total RNAs from stomach tumors were isolated with TRIzol only, because the tumors were small and only very small amounts of total RNAs were extracted.

FDD FDD was performed according to the procedures described previously.[15] In brief, total RNAs (2.5 μg) were reverse-transcribed with a fluorescein isothiocyanate (FITC)-labeled 3′-anchored oligo(dT) primer ($GT_{15}C$) and SuperScript II reverse transcriptase (Life Technologies, Inc.). First strand cDNA, corresponding to 50 ng of total RNA, was used for PCR reaction with 1 unit of *Taq* DNA polymerase, 10 pmol of arbitrary 5′ primer (OPB01, 5′-GTTTCGCTCC-3′; Operon Technologies, Inc., Alameda, CA), and 5 pmol of 3′-anchored primer, $GT_{15}C$. The mixture was subjected to a low stringency PCR. PCR products were fractionated on a 5% denaturing acrylamide gel and visualized by scanning with a fluorescence image analyzer (FluorImager 575; Molecular Dynamics, Sunnyvate, CA). Reamplified products were cloned into the deoxyT-added *Eco*RV site of pT7Blue vector (Novagen, Madison, WI). The selection of clones by the precise sizing of the inserts, and DNA sequencing, were performed according to the procedures described previously.[3]

RT-PCR and competitive RT-PCR RT-PCR and competitive RT-PCR with specific primers and the first-strand cDNA were performed at high stringency.[7] The competitors were prepared by RT-PCR with specific primers as described below and purified with 5% polyacrylamide gel electrophoresis. RT-PCR was performed for 25 cycles with specific primers in the presence of an appropriate amount of competitor. The amplified cDNAs were examined on a 5% polyacrylamide gel stained with SYBR Green I (Molecular Probes, Eugene, OR) and scanned with a fluorescence image analyzer. The densitogram was determined with the same instrument. The respective band area in the MNNG-exposed group lane was compared with the same size area in the control group lane. cDNA sequence data were obtained from an internet website (http://www.ncbi.nlm.nih.gov/). The specific oligonucleotide primers were as follows: osteonectin, 5′-CTGCA-

GAAGAGATGGTGGCGG-3′ (5′ primer) and 5′-CAGGCAGGGGGCAATGTATTTG-3′(3′ primer) (395 bp); collagen type III, 5′-GGACCTGCTGGTCCTCCTGG-3′ (5′ primer) and 5′-GCCTGGTGGTCCAGCAAGACC-3′ (3′ primer) (345 bp); fibronectin, 5′-CCCTGTATGCTGTCACTGGCC-3′ (5′ primer) and 5′-GCAGTCTGAACCAGGGGCTGG-3′ (3′ primer) (334 bp); osteopontin, 5′-GGAGGAGAAGGCGCATTACAGC-3′ (5′ primer) and 5′-GGACTTTGACCTCAGTCCATAAGC-3′ (3′ primer) (388 bp); proteoglycan NG2, 5′-GGCCAAACAGATCATCTCCTGC-3′ (5′ primer) and 5′-CCAGGCCAACTTCATCACCAGC-3′ (3′ primer) (412 bp); laminin γ1, 5′-CGACGCTGCCAAGGCTCTTGC-3′ (5′ primer) and 5′-CCATCATCATGTCCTGGTCGGC-3′ (3′ primer) (438 bp); S-laminin, 5′-CGAGGAAGCTGCTATCCAGCC-3′ (5′ primer) and 5′-CCGAAGTCTGCAGAGCGCTCC-3′ (3′ primer) (377 bp); mouse 18S rRNA, 5′-CCAGTAAGTGCGGGTCATAAGC-3′ (5′ primer) and 5′-CCTTCCGCAGGTTCACCTACG-3′ (3′ primer) (230 bp). The competitor for osteonectin was prepared by RT-PCR with 42mer 5′ primer 5′-CTGCAGAAGAGATGGTGGCGGCCCAGTCCAGGTGGAAATGGG-3′ and 3′ primer for osteonectin (352 bp). The competitor for collagen type III was prepared by RT-PCR with 41mer 3′ primer 5′-GCCTGGTGGTCCAGCAAGACCCCTGGAGGACCCTGAGCACC-3′ and 5′ primer for collagen type III (301 bp). The competitor for fibronectin was prepared by RT-PCR with 44mer 3′ primer 5′-GCAGTCTGAACCAGGGGCTGGCCACATACTCCACGGTGGGTTGC-3′ and 5′ primer for fibronectin (299 bp). The competitor for osteopontin was prepared by RT-PCR with 47mer 3′ primer 5′-GGACTTTGACCTCAGTCCATAAGCGCGATTGGAGTCAAAACGTCTGC-3′ and 5′ primer for osteopontin (347 bp). The competitor for proteoglycan NG2 was prepared by RT-PCR with 46mer 5′ primer 5′-GGCCAAACAGATCATCTCCTGCGGGACAAAATGAGCTGAGTCTGC-3′ and 3′ primer for proteoglycan (371 bp). The competitor for laminin γ1 was prepared by RT-PCR with 45mer 5′ primer 5′-CGACGCTGCCAAGGCTCTTGCCGAGTGAACGATAACAAGACAGC-3′ and 3′ primer for laminin γ1 (358 bp). The competitor for S-laminin was prepared by RT-PCR with 42mer 5′ primer 5′-CGAGGAAGCTGCTATCCAGCCCCTCCTCCACGTGTGGCTTGC-3′ and 3′ primer for S-laminin (336 bp).

Northern blot analysis For northern blot analysis, total RNAs (50 μg) were fractionated on a formaldehyde agarose gel, transferred to a positively charged Hybond N+ nylon membrane (Amersham-Pharmacia Biotech, Buckinghamshire, UK), and hybridized with ^{32}P-labeled probe. The membranes were washed and exposed on an imaging plate, and the image was analyzed with a BAS 2000A (Fuji Film, Tokyo) as described previously.[7)]

Immunohistochemistry and BrdU-labeling Immunohistochemical staining was performed with monoclonal antibodies on five rats in each group as described previously.[16)] Five sections from each rat were stained and analyzed. Areas containing severe erosions were avoided for photographs. Monoclonal antibody against human osteonectin (H95031M) was purchased from Biodesign Inter-

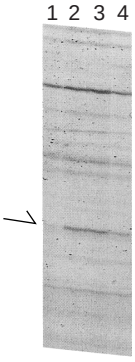

Fig. 1. Fluorescent differential display of PCR products from cDNA derived from total RNA of Lewis rat stomach pyloric mucosa. Lanes 1–3 : 0, 14 and 27-day MNNG exposure. Lane 4 : 27-day MNNG exposure and 7-day water drinking. The cDNA band of 125 bp (arrow) increased severalfold 14 and 27 days after the beginning of MNNG exposure and decreased 7 days after the cessation.

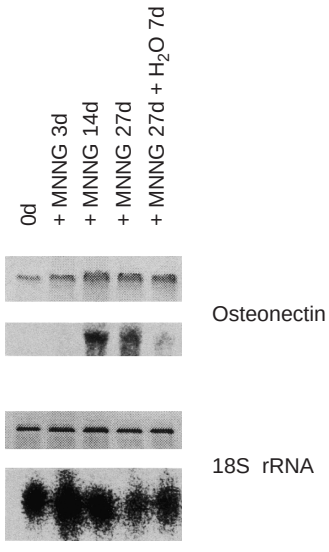

Fig. 2. RT-PCR (upper lane) and northern blot analysis (lower lane) of osteonectin in Lewis rat stomach pyloric mucosa. 18S rRNA shows the relative amount of RT-cDNA and total RNA. Rats were exposed to MNNG (100 mg/liter) for 0, 3, 14 and 27 days. The fifth group was examined after 27 days of MNNG and 7 days of water.

national (Kennebunk, ME). The cellular kinetics of stomach pyloric mucosa were studied by immunohistochemical staining with BrdU as described previously.[17] The numbers of total and BrdU-positive cells were counted for 100 pyloric glands and the percentage of positive cells was calculated.

RESULTS

Cloning of osteonectin FDD of cDNA from stomach pyloric mucosa shows that the band of 125 bp increased severalfold 14 and 27 days after the beginning of MNNG exposure and decreased 7 days after the cessation (Fig. 1). The band was cloned and sequenced. The sequence was identical to the 3′ end of osteonectin (1915–2017 bases from the 5′ origin of rat osteonectin cDNA, gi2196883), an extracellular matrix-associated matricellular glycoprotein.

RT-PCR and northern blot of osteonectin in stomach pyloric mucosa Fig. 2 shows the results of RT-PCR with specific primers of osteonectin and northern blot. The gene expression of osteonectin was low in the pyloric mucosa of control animals (0 d) and of animals after 3 days of MNNG exposure. A severalfold increase in expression was observed in pyloric mucosa with 14 and 27 days of MNNG exposure. The expression decreased 7 days after the cessation of MNNG exposure (MNNG 27d + H_2O 7d), as detected in the northern blot.

Immunohistochemical demonstration of osteonectin in stomach pyloric mucosa Fig. 3, a and b shows immunohistochemical staining of pyloric mucosa with an osteonectin-specific mAb. This demonstrates that the expression of osteonectin was undetectable in control tissue, and that osteonectin-mAb-stained fibroblastic cells appeared in interstitial tissue at the base of pyloric mucosa after 14 days of MNNG exposure.

Competitive RT-PCR of other extracellular matrix protein genes in stomach pyloric mucosa Gene expression of six other extracellular matrix proteins was also examined. Fig. 4 shows that six extracellular matrix protein genes generally had low expression in pyloric mucosa of control animals (0 d). MNNG exposure for 14 days induced increased expression of these genes: collagen type III, 32-fold; fibronectin, 19-fold; osteopontin, 6-fold, proteoglycan NG2, 23-fold; laminin γ1, 6-fold; S-laminin, 5.5-fold. Thus, osteonectin was not the only extracellular matrix protein gene with expression induced by MNNG. Seven days after cessation of a 27-day MNNG exposure, the levels had generally decreased slightly, if at all.

Cell proliferation Cell proliferation, measured in terms of BrdU-labeling indices, was studied in the stomach

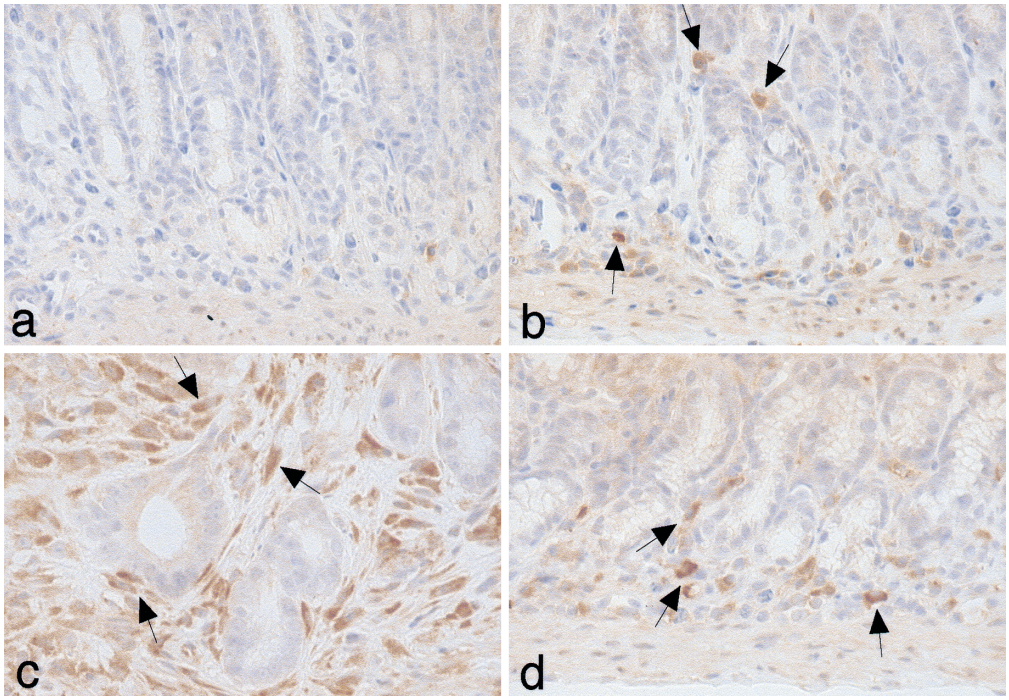

Fig. 3. Immunohistochemical staining of osteonectin in the pyloric mucosa and in the stomach tumor. Control (a) and 14-day MNNG-exposed (b) pyloric mucosa. The stomach tumor (T2) induced by MNNG (c) and non-tumorous pyloric mucosa in a stomach tumor-bearing rat (d). Brown cells (arrow) are mAb-stained cells in interstitial tissue. ×200.

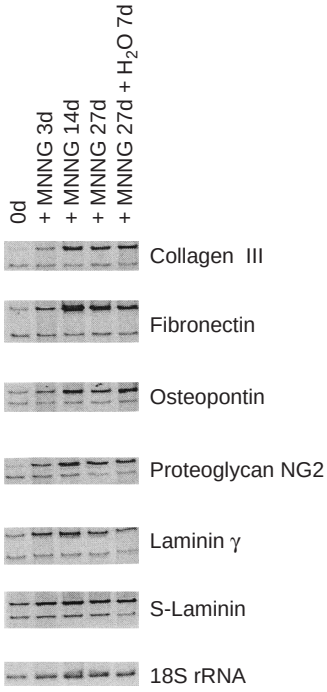

Fig. 4. Competitive RT-PCR of six extracellular matrix protein genes (collagen type III, fibronectin, osteopontin, proteoglycan NG2, laminin γ1 and S-laminin) in rat stomach pyloric mucosa. Shown are the target cDNA product (upper band) and competitor (lower band); 18S rRNA shows the relative amount of RT-cDNA.

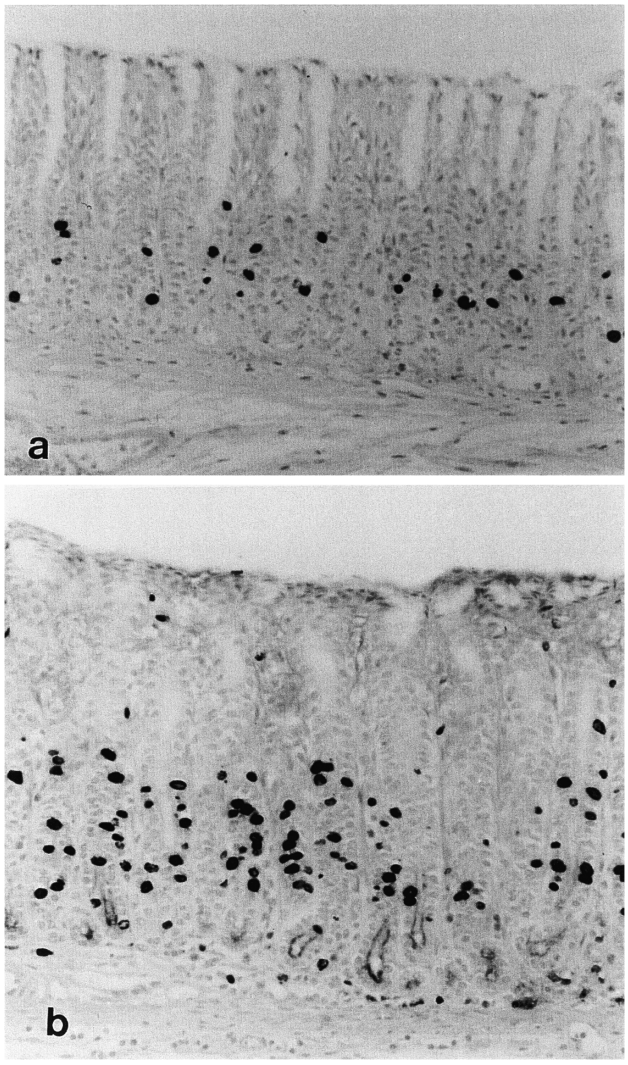

Fig. 5. BrdU staining in the stomach pyloric mucosa. Samples are control (a) and 14-day MNNG-exposed (b) rats. Dark cells are BrdU-stained cells. ×200.

pyloric mucosa after MNNG exposure. Fig. 5 and Table I show that a threefold increase in cell proliferation was induced after 14 days of MNNG exposure.

No increase in osteonectin or extracellular matrix protein gene expression by NaCl, a stomach tumor promoter Fig. 6 shows that NaCl, a glandular stomach tumor promoter, did not increase gene expression of osteonectin and six extracellular matrix proteins. However, in confirmation of the previous results, the carcinogen MNNG increased gene expression of osteonectin and the six extracellular matrix proteins.

Competitive RT-PCR of osteonectin in stomach tumors Increased gene expression of osteonectin was demonstrated by competitive RT-PCR in twelve stomach adenocarcinomas and adenomas (Fig. 7). The maximum increase was in T9 (34-fold) and the minimum in T3 (6-fold). The controls showed low osteonectin expression without MNNG, while expression was induced with 14 days of MNNG exposure.

Immunohistochemical demonstration of osteonectin in stomach tumors Fig. 3c demonstrates that the expression of osteonectin is evident in fibroblastic cells in interstitial tissue of stomach tumor induced by MNNG. Fig. 3d shows stained cells in interstitial tissue of non-tumorous pyloric mucosa in a stomach tumor-bearing rat.

Competitive RT-PCR of other extracellular matrix protein genes in stomach tumors The expression of six other extracellular matrix protein genes generally increased in almost all stomach tumors versus the respective controls (Lewis control or WKY control) (Fig. 8). The greatest increases in expression of individual genes were as follows: collagen type III (in T9, 54-fold), fibronectin (in T11, 138-fold), osteopontin (in T9, 12-fold), proteoglycan NG2 (in T9, 70-fold), laminin γ1 (in T11, 16-fold) and S-laminin (in T9, 7.1-fold). The minimum increases detected were: for collagen type III, 7-fold (T3); fibronec-

Table I. Increase in Labeling Indices with BrdU in the Stomach Pyloric Mucosa after 14 Days of MNNG Exposure

	n	Labeling index (%)
Control	4	2.04±0.23
MNNG-exposed	4	6.48±0.18[a)]

a) Significantly different from control at $P<0.001$.
Male 6-week-old rats were given MNNG (100 mg/liter) in the drinking water for 14 days. Control rats were given distilled water. BrdU was injected 1 h before killing. Stomachs were removed and fixed for histological examination, and thin sections were stained histochemically with BrdU-mAb. One hundred pyloric glands were scored for each rat.

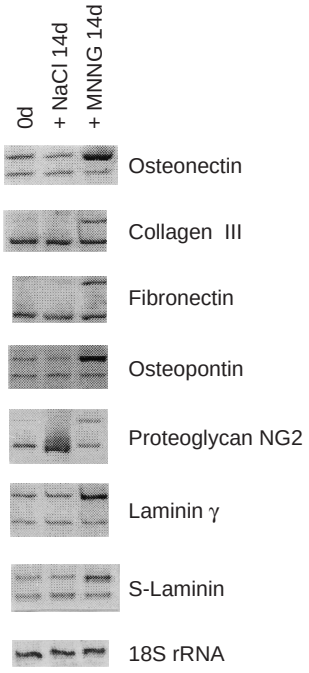

Fig. 6. Expression of osteonectin and extracellular matrix protein genes in Lewis rat stomach pyloric mucosa after a 14-day dietary exposure to NaCl, a stomach tumor promoter. Competitive RT-PCR was conducted as described in "Materials and Methods." Shown are target cDNA product (upper band) and competitor (lower band). Samples from MNNG-exposed rats are shown as positive controls.

tin, 4.9-fold (T6); osteopontin, 4.2-fold (T10); proteoglycan NG2, 7.5-fold (T6); laminin γ1, 2.2-fold (T6); and for S-laminin, 1.5-fold (T1).

DISCUSSION

In this study, we found the appearance of osteonectin-expressing fibroblastic cells in interstitial tissue of stomach pyloric mucosa in the early initiation step of MNNG-

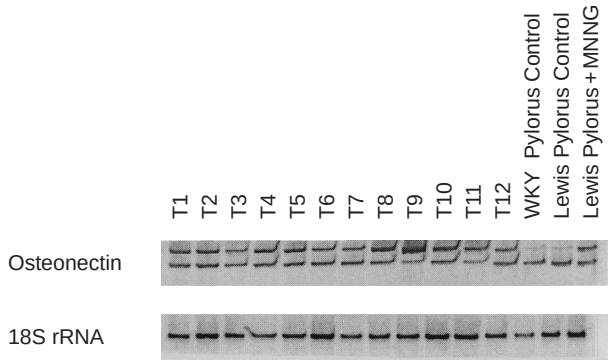

Fig. 7. Competitive RT-PCR of osteonectin in stomach tumors induced in Lewis rats (T2–5, adenocarcinomas; T1 and T6, adenomas) and WKY rats (T7–12, adenocarcinomas) by MNNG, and control WKY and Lewis stomach pyloric mucosa (negative controls) and Lewis stomach pyloric mucosa after 14 days of MNNG exposure (positive control). The lower bands were competitors. 18S ribosomal RNA shows the relative amount of RT-cDNA.

induced rat stomach carcinogenesis. The expression of osteonectin was evident in fibroblastic cells in interstitial tissues of stomach adenocarcinomas and adenomas induced by MNNG. Appearance of osteonectin-expressing fibroblastic cells in interstitial tissue of stomach pyloric mucosa is suggested to be associated with carcinogenesis. These are the first data showing that osteonectin is associated with rat stomach carcinogenesis. A severalfold increase in expression of osteonectin was observed not only in Lewis rats, but also in MNNG-sensitive ACI rats (data not shown). Although the number of tumors examined was rather small, all results were similar and showed positive expression of osteonectin. Thus, it appears that the present results are typical in differentiated stomach adenocarcinomas and adenomas induced with MNNG. We examined the expression of osteonectin in the fundic and duodenum mucosa in Lewis rats by RT-PCR. A significant increase such as that in the pyloric mucosa was not observed in these tissues after 14 days of MNNG exposure (data not shown).

Additionally we found that expression of several extracellular matrix protein genes increased and that the elevated expression continued until tumor development. It has been postulated that changes in extracellular matrix proteins and associated proteins play a key role in the process of tumoral invasion and metastasis in certain malignancies. However, the present results suggest that such changes begin to occur in the early initiation step of carcinogenesis and probably play a role in carcinogenesis itself.

Alterations in extracellular matrix, cell-cell and cell-matrix adhesion, and oncogenes are thought to be impor-

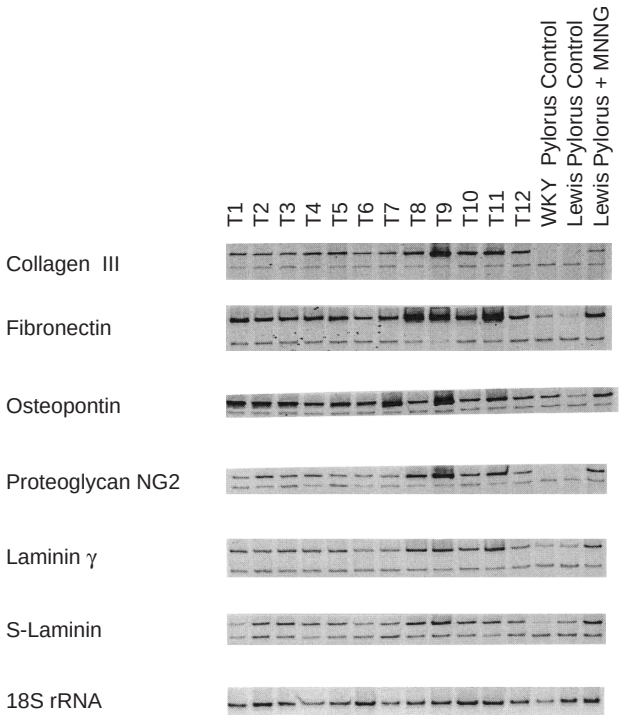

Fig. 8. Competitive RT-PCR of six extracellular matrix protein genes (collagen type III, fibronectin, osteopontin, proteoglycan NG2, laminin γ1 and S-laminin) in stomach tumors. Shown are the target cDNA product (upper band) and competitor (lower band). 18S rRNA shows the relative amount of RT-cDNA.

tant in tumor progression and metastasis.[18] Several reports document the overexpression of collagen type III,[19] fibronectin[20] and osteopontin[21] in human stomach cancers. Expression of these genes was often observed in cells in tumor stroma and sometimes in tumor cells. Overexpression of proteoglycan NG2 was reported in various cancers[22] but little is known about the situation in stomach cancer. There are many reports on laminin[23] in cancers, but little is known about laminin γ1 and S-laminin in stomach cancer. The precise role of the extracellular matrix in cancer development has not been elucidated. However, characterization of the transcriptional regulation of these extracellular matrix protein genes and the functions of the proteins should provide a penetrating insight into the regulatory interactions that have been perturbed in cancer.

Recently we examined osteonectin expression in a considerable number of human stomach cancer tissues immunohistochemically and molecular-biologically. Osteonectin-expressing cells were often observed in interstitial tissue of differentiated adenocarcinomas and moderately often observed in undifferentiated adenocarcinomas.[24] The results support our suggestion that osteonectin-expressing cells in interstitial tissue play a role in development of differentiated adenocarcinomas.

Recently, antisense RNA as a selective inhibitor of osteonectin has been proposed.[25] Down-regulation of osteonectin prevented tumor formation in nude mice, a procedure that appears to be safer than the use of traditional anti-carcinogenesis drugs. These results suggest the significance of osteonectin in tumor formation.

The present results and previous reports of dendritic cell appearance[6, 7] document the changes in the interstitial tissue of rat pyloric mucosa in the early phase of chemical carcinogenesis. Induction of pepsinogen-altered pyloric glands by MNNG[4, 17] showed early changes in epithelial cells of pyloric mucosa. Thus, various changes are induced both in epithelial cells and interstitial tissue of pyloric mucosa in the early initiation stage and these changes continued until tumor development after 12 months.

In conclusion, we found that osteonectin-expressing cells appear in the interstitial tissue of pyloric mucosa from the initiation stage of rat stomach chemical carcinogenesis and that the elevated expression continues until tumor development. These changes perhaps play a role in cancer development.

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