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Dendritic Cell Appearance and Differentiation during Early and Late Stages of Rat Stomach Carcinogenesis

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Dendritic cell appearance and differentiation during early and late stages of rat stomach carcinogenesis were studied in the pyloric mucosa. Young male rats were given drinking water with or without N-methyl-N′-nitro-N-nitrosoguanidine (MNNG; 100 mg/liter) for 14 days. Use of competitive RT-PCR and northern blotting showed that MNNG exposure induced 3- to 4-fold greater expression of the genes for *integrin β7* and *integrin αE2* (identical with antigen *OX-62*, a dendritic cell marker), as well as three cytokines, *IL-4*, *GM-CSF* and *TNFα*, in the stomach pyloric mucosa of resistant Buffalo rats compared to sensitive ACI rats. These genes were minimally expressed in control animals. The results confirm the appearance of dendritic cells in the target pyloric mucosa and suggest the possibility that dendritic cell differentiation and maturation are induced by various cytokines, at least in Buffalo rats. Competitive RT-PCR showed expression of *integrin αE2* and *β7*, MHC class II-associated invariant chain (*Ii*), *MHC class II*, *B7-1*, *CD28*, *GM-CSF* and *TNFα* genes in all 12 examined stomach adenocarcinomas and adenomas induced in male Lewis and WKY rats with 30 weeks' MNNG exposure, suggesting the presence of dendritic cells in tumors. *OX-62* staining and western blotting for *OX-62* also confirmed the presence of dendritic cells in tumors. However, the population of dendritic cells in tumors was less than that in the pyloric mucosa after 14 days' MNNG exposure. The present results suggest that immune defense involving dendritic cells is marshaled from the very early initiation stage during rat stomach cancer development, but is downgraded in developed tumors.

Key words: Dendritic cell — Cytokines — Rat stomach pyloric mucosa — MNNG

There have been few studies on the involvement of the immune response in the initiation stage of chemical carcinogenesis.[1–3] Previously we studied rat experimental stomach carcinogenesis induced with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)[4] to clarify gene expression changes during the initiation stage by fluorescent differential display analysis.[5] With cloned MHC class II-associated invariant chain (*Ii*) from the target organ, stomach pyloric mucosa, of male Lewis rats,[5] we found that administration of 100 mg/liter MNNG in drinking water for 8 days induced the appearance of *Ii* expression. The up-regulation of *Ii* in this organ was demonstrated by reverse transcription-PCR and northern blotting. Immunohistochemical staining revealed that *Ii*-monoclonal antibody (mAb)-stained cells appeared in interstitial tissue in the pyloric mucosa 14 days after the beginning of MNNG exposure.

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Recently we reported that *Ii*-mAb-stained cells would also stain with *OX-62* mAb (a marker of dendritic cells[6]).[7] Thus, dendritic cells, antigen-presenting cells, were shown to appear in the interstitial tissue of stomach pyloric mucosa 14 days after MNNG exposure. Furthermore, we compared the appearance of dendritic cells in a carcinogenesis-resistant rat strain (Buffalo) and a sensitive strain (ACI).

Different susceptibility to MNNG-induced stomach cancer was reported in Buffalo and ACI rats.[8] The resistance in Buffalo rats was genetically determined and autosomaly dominant.[9] A greater number of mature dendritic cells expressing *MHC class II*, *Ii*, *MHC class I*, *B7-1* and *B7-2* appeared in the interstitial tissue of pyloric mucosa of the resistant Buffalo rats. This suggested the involvement of a dendritic cell response in the resistance to the MNNG induction of stomach carcinogenesis in rats.[7] The presence of a susceptibility gene and two resistance genes was suggested,[10] but the relationships of these genes to dendritic cells were not elucidated.

The *OX-62* gene was recently cloned and the gene product was identified as *integrin αE2*[11]; the DNA sequence

was released onto DNA databases in 1998. *Integrin β7* is another unit of the hetero-duplex integrin complex containing *integrin αE2*.[12] Thus, the expression of *integrin αE2* and *β7* genes was examined in the present study. E-Cadherin, a cell adhesion molecule, is a ligand of the integrin αE2 and β7 complex,[12] so the expression of *E-cadherin* was also examined in the present study.

Multiple pathways for dendritic cell differentiation and maturation have been proposed, mainly from *in vitro* studies.[13–17] There are at least two possibilities for the source of dendritic cells in the stomach pyloric mucosa. First, dendritic cells may be formed by differentiation and maturation of precursor cells within the tissue by the action of cytokines. Second, mature dendritic cells may migrate from other sources. The first possibility is examined in the present study.

We also examined the presence of dendritic cells in rat stomach tumors to determine whether the appearance of dendritic cells in the target organ is a continuous phenomenon or a temporary event associated only with the early stages of rat stomach carcinogenesis.

MATERIALS AND METHODS

Animals Ten male 6-week-old Buffalo rats (BUF/NacJcl; Nihon Clea, Tokyo), ACI rats (ACI/NJcl; Nihon Clea) and Lewis rats (LEW/Crj; Charles River Japan, Inc., Yokohama) 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. Ten control rats were given distilled water instead of MNNG. Rats had free access to pellet rodent chow (CE-2, Nihon Clea). 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. Stomachs from the other five rats were washed with cold phosphate-buffered saline, 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) (Nacalai Tesque, Inc.) 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 cut off 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.

RNA and protein isolations Total RNAs from stomach pyloric mucosa and tumors 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.[18] Total RNAs from stomach tumors were isolated with only TRIzol, because the tumors were small and only small amounts of total RNAs were extracted. Proteins were extracted from the residue as described in the ISOGEN LS manual.

Competitive RT-PCR The first cDNA strand was prepared with 2.5 μg of total RNA and 50 pmol of $GT_{15}C$ by using SuperScript II reverse transcriptase (Life Technologies, Inc.). Competitive RT-PCR with specific primers and the first-strand cDNA was performed at high stringency.[18] 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 (FluorImager 575; Molecular Dynamics, Sunnyvale, CA). The densitogram was obtained 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. To compare MNNG-exposed Buffalo rats with ACI rats, the respective band areas of electrophoresed samples from MNNG-exposed animals were compared. cDNA sequence data were obtained from an internet website (http://www.ncbi.nlm.nih.gov/). The oligonucleotide primers were as follows: *integrin αE2*, 5′-GGACATCAACGCCTCCCTTGC-3′ (5′ primer) and 5′-GGTGTCTCCAACTGTGCCTTCC-3′ (3′ primer) (409 bp); *integrin β7*, 5′-GGTACGGGTCCCCTGGCAGC-3′ (5′ primer) and 5′-GGGTTGACGGTAGTTGTGACCG-3′ (3′ primer) (389 bp); *E-cadherin*, 5′-GGCCTTGATGCCAGACCGGAAG-3′ (5′ primer) and 5′-CCACTCCCCTCATAGTCAAACACC-3′ (3′ primer) (194 bp); *IL-4*, 5′-GGGTCTCAGCCCCCACCTTGC-3′ (5′ primer) and 5′-GCAGCTTCTCAGTGAGTTCAGACC-3′ (3′ primer) (341 bp); *GM-CSF*, 5′-GCTCACCCAACCCTGTCACCCG-3′ (5′ primer) and 5′-CCTCATTTCTGGACCGGCTTCC-3′ (3′ primer) (376 bp); *TNFα*, 5′-GCAGCTGGAGTGGCTGAGCC-3′ (5′ primer) and 5′-GCAATGACTCCAAAGTAGACCTGC-3′ (3′ primer) (393 bp); *Ii*, 5′-CTCTGTCCTGGTGGCTCTGCTCTT-3′ (5′ primer) and 5′-AGTCTGGGTGGGCTGCTTCTCCTC-3′ (3′ primer) (457 bp); *MHC class II*, 5′-GTCTGGTAGGCATCGTCGTC-3′ (5′ primer) and 5′-TGGAGAAATGTCAAGCCGTAAGTG-3′ (3′ primer) (400 bp); *B7-1*, 5′-CCGGGGTACCGGAAGTGTGG-3′ (5′ primer) and 5′-CGTCGCGTTGAAGTCTAGTTGG-3′ (3′ primer) (395 bp); *CD28*, 5′-GGTGAAGCAGTCCCCGCTGCC-3′ (5′ primer) and 5′-CCAGCAACCACGACCAGTGGC-3′ (3′ primer) (408 bp); and mouse *18S rRNA*, 5′-CCAGTAAGTGCGGGTCATAAGC-3′ (5′ primer) and 5′-CCTTCCGCAGGTTCACCTACG-3′ (3′ primer) (218 bp). The competitor for *integrin αE2* was prepared by RT-PCR with 42 mer-5′ primer 5′-GGACATCAACGCCTCCCTTGCCCT-

CAGCCATGCAGCATGTCC-3′ and with 3′ primer for *integrin αE2* (365 bp). The competitor for *integrin β7* was prepared by RT-PCR with 41 mer-5′ primer 5′-GGTACGGGTCCCCTGGCAGCGGCCCCCAATTTGGATGATGG-3′ and with 3′ primer for *integrin β7* (344 bp). The competitor for *E-cadherin* was prepared by RT-PCR with 42 mer-5′ primer 5′-GGCCTTGATGCCAGACCGGAAGCGTCCCCGTCCAGCCAATCC-3′ and with 3′ primer for *E-cadherin* (147 bp). The competitor for *IL-4* was prepared by RT-PCR with 42 mer-5′ primer 5′-GGGTCTCAGCCCCCACCTTGCCCACGGATGTAACGACAGCCC-3′ and with 3′ primer for *IL-4* (299 bp). The competitor for *GM-CSF* was prepared by RT-PCR with 44 mer-5′ primer 5′-GCTCACCCAACCCTGTCACCCGGCCTCCTAAATGACATGCGTGC-3′ and with 3′ primer for *GM-CSF* (338 bp). The competitor for *TNFα* was prepared by RT-PCR with 43 mer-5′ primer 5′-GCAGCTGGAGTGGCTGAGCCGGTACCAGCAGATGGGCTGTACC-3′ and with 3′ primer for *TNFα* (338 bp). The competitor for *Ii* was prepared by RT-PCR with 48 mer-3′ primer 5′-AGTCTGGGTGGGCTGCTTCTCCTCCTTAAGGTGCTTCAGATTCTCCGG-3′ and 5′ primer for *Ii* (373 bp). The competitor for *MHC class II* was prepared by RT-PCR with 47 mer-3′ primer 5′-TGGAGAAATGTCAAGCCGTAAGTGCTCTGTGATATGTGCCACAGAGG-3′ and 5′ primer for *MHC class II* (353 bp). The competitor for *B7-1* was prepared by RT-PCR with 42 mer-5′ primer 5′-CCGGGGTACCGGAAGTGTGGCCTGATCCTTTCAGACAGGGGC-3′ and 3′ primer for *B7-1* (333 bp). The competitor for *CD28* was prepared by RT-PCR with 41 mer-5′ primer 5′-GGTGAAGCAGTCCCCGCTGCGCAAAGGAGTTCCGGGCATCC-3′ and with 3′ primer for *CD28* (342 bp).

Northern blot Total RNAs (50 μg) were resolved on a 1% agarose-2.2 *M* formaldehyde gel, blotted onto a Hybond-N membrane filter (Amersham, Buckinghamshire, UK), and then hybridized with a ^{32}P-labeled probe. Washed filters were exposed on an imaging plate and analyzed with a BAS 2000 (Fuji Film, Tokyo). The densitogram was determined with the same instrument in the same manner as for competitive RT-PCR.

Western blot Protein fractions were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, blotted onto an Immobilion polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) and stained by alkaline phosphatase-linked immunostaining.[19] *OX-62* mAb (dendritic cell marker; Pharmingen, San Diego, CA) was used at 1/1000 dilution. Monoclonal anti-*β-actin* (clone AC-15, Sigma, Saint Louis, MO) was used at 1/1000 dilution to show the relative amount of protein.

Histochemistry Immunohistochemical staining was per-

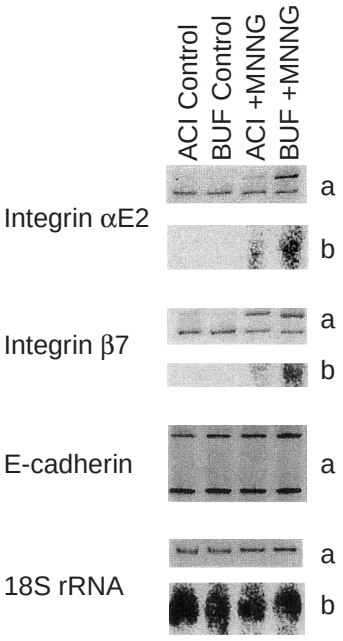

Fig. 1. Competitive RT-PCR (a) and northern blot (b) of *integrin αE2* and *β7* and *E-cadherin* in stomach pyloric mucosa of MNNG-sensitive ACI rats and resistant Buffalo rats, 14 days after MNNG exposure in the drinking water. Samples from the left were control ACI (ACI Control), control Buffalo (BUF Control), MNNG-exposed ACI (ACI +MNNG) and MNNG-exposed Buffalo (BUF +MNNG). a: The lower bands in competitive RT-PCR were competitors. b: Total RNAs for northern blot were 50 μg/lane. 18S rRNA shows the relative amounts of RT-cDNA and total RNA.

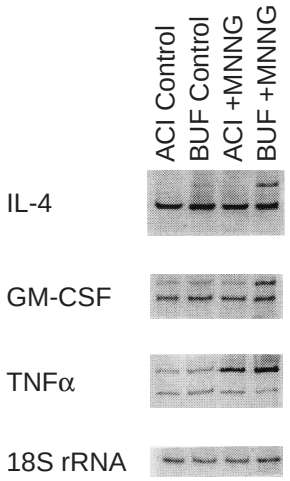

Fig. 2. Competitive RT-PCR of *IL-4*, *GM-SCF* and *TNFα* in stomach pyloric mucosa of MNNG-resistant Buffalo and -sensitive ACI rats, 14 days after MNNG exposure in the drinking water. Samples were the same as in Fig. 1. The lower bands were competitors. 18S rRNA shows the relative amount of RT-cDNA.

formed on tissue from 5 rats in each group using monoclonal antibodies as described previously.[20] Five tissue sections from each rat were stained and analyzed. Areas containing severe erosion were avoided for photography. *OX-62* mAb (Pharmingen) was used on frozen sections fixed with acetone. *Ii*-mAb RG11 directed against the carboxyl-terminal segment of the rat invariant γ chain was a gift from K. Reske.[21] Antibody against human *E-cadherin* (TL-C20820) was obtained from Transduction Laboratories (Lexington, KY). These antibodies were used on ethanol-fixed paraffin sections.

RESULTS

Increase in expression of *integrin αE2* and *integrin β7* in stomach pyloric mucosa The results of competitive RT-PCR and northern blotting of *integrin αE2* and *integrin β7* genes in stomach pyloric mucosa are shown in Fig. 1. The expression of these genes was weak or undetectable in pyloric mucosa of control animals. MNNG exposure for 14 days induced 100- and 20-fold increases in the expression of *integrin*s *αE2* and *β7*, respectively, in the pyloric mucosa of the resistant Buffalo rats, but 4- and

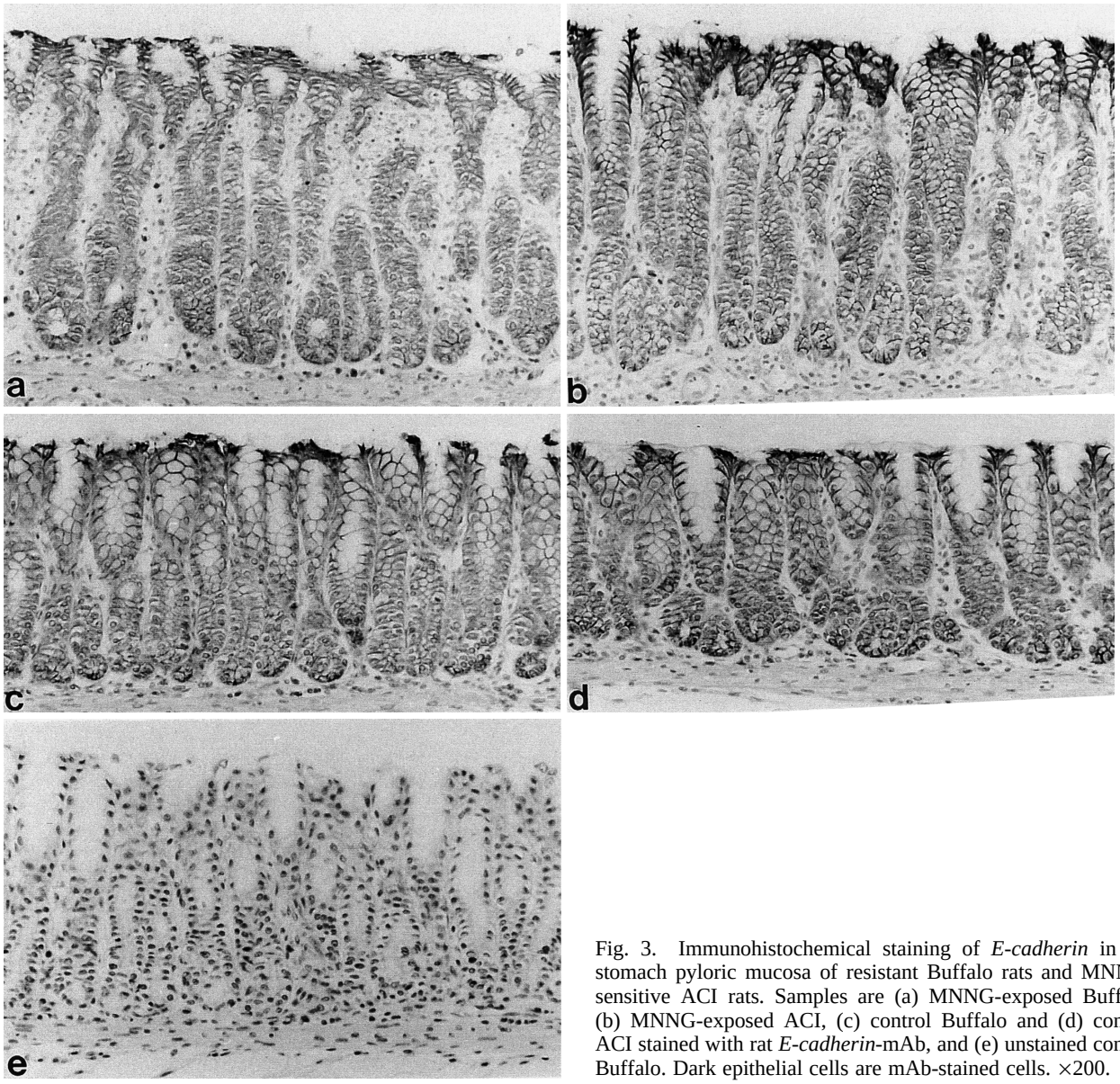

Fig. 3. Immunohistochemical staining of *E-cadherin* in the stomach pyloric mucosa of resistant Buffalo rats and MNNG-sensitive ACI rats. Samples are (a) MNNG-exposed Buffalo, (b) MNNG-exposed ACI, (c) control Buffalo and (d) control ACI stained with rat *E-cadherin*-mAb, and (e) unstained control Buffalo. Dark epithelial cells are mAb-stained cells. ×200.

10-fold increases in the sensitive ACI rats in northern blots. However, the expression of *integrin*s *αE2* and *β7* in the MNNG-exposed pyloric mucosa of the resistant Buffalo rats was 3-fold greater than that of the sensitive ACI rats. The present results are consistent with the previous findings[7] that a greater number of *OX-62*-stained cells appeared in the interstitial tissue of the resistant Buffalo rats than in sensitive ACI rats.

Increase in expression of *IL-4*, *GM-CSF* and *TNFα* genes in stomach pyloric mucosa The results of competitive RT-PCR of *IL-4*, *GM-CSF* and *TNFα* genes in stomach pyloric mucosa are presented in Fig. 2. This shows that expression of these genes was weak or undetectable in pyloric mucosa of control animals. MNNG exposure for 14 days induced a 4-fold increase in *IL-4* and *GM-CSF* expression in Buffalo rats, but no increase was detected in ACI rats. Expression of *TNFα* was increased 10-fold in both Buffalo and ACI rats. *IL-4*, *GM-CSF* and *TNFα* were reported as inducers of dendritic cell differentiation and maturation *in vitro* in humans and rats.[13, 15, 22] The present results suggest the possibility that dendritic cells were matured in the stomach pyloric mucosa from peripheral blood precursor cells by *IL-4*, *GM-CSF* and *TNFα*, at least in Buffalo rats.

Expression of *E-cadherin* gene in stomach pyloric mucosa E-Cadherin is a ligand of the integrin αE2 and β7 complex.[12] The results of competitive RT-PCR and northern blotting of the *E-cadherin* gene in stomach pyloric mucosa are shown in Fig. 1. The gene was expressed in pyloric mucosa of control animals, with no change after MNNG exposure.

Immunohistochemical demonstration of *E-cadherin* in stomach pyloric mucosa In order to examine *E-cadherin* gene expression at the protein level, immunohistochemical staining of pyloric mucosa was conducted with an *E-cadherin*-specific mAb. Representative results are shown in Fig. 3. Cell membranes of the pyloric mucosa were stained with the antibody in both control and MNNG-exposed ani-

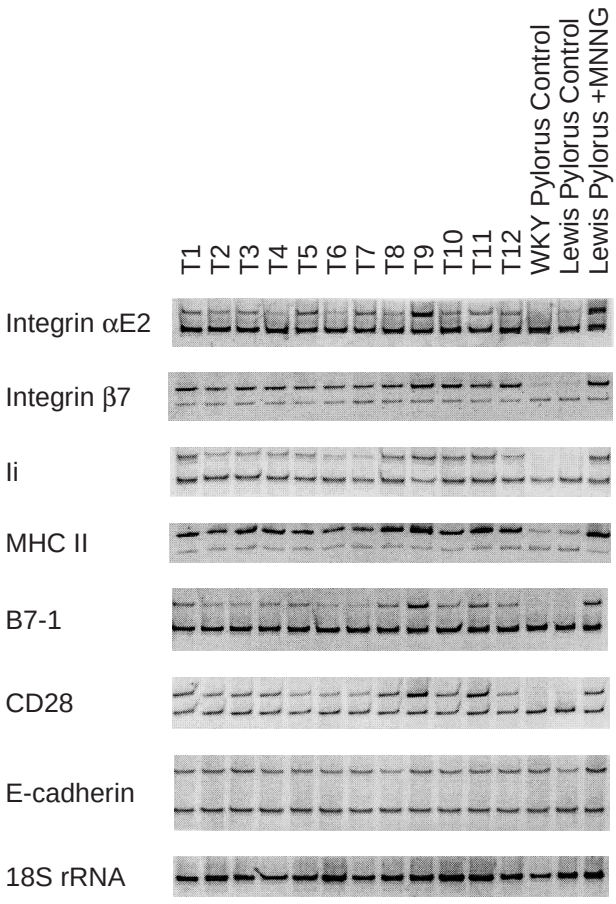

Fig. 4. Competitive RT-PCR of *integrin αE2* and *β7*, *Ii*, *MHC class II*, *B7-1*, *CD28* and *E-cadherin* in stomach tumors induced in Lewis rats (T2–5, adenocarcinomas; T1 and T6, adenomas) and WKY rats (T7–12, adenocarcinomas) by MNNG, and control WKY and Lewis stomach pyloric mucosa (negative controls) and Lewis pyloric mucosa after 14 days' MNNG exposure (positive control). The lower bands were competitors. 18S ribosomal RNA shows the relative amount of RT-cDNA.

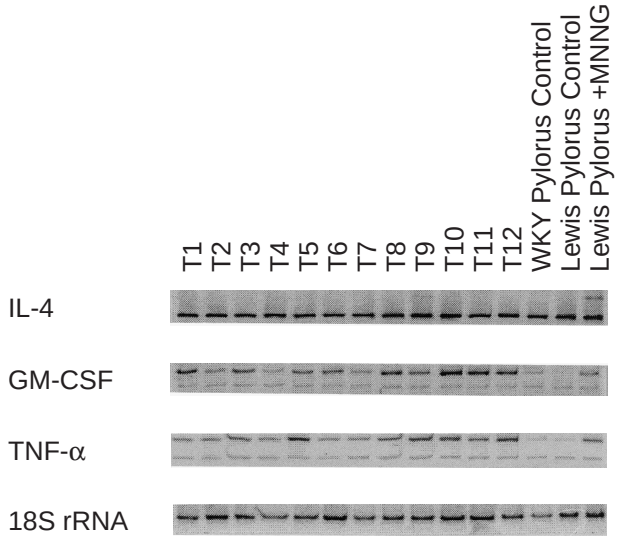

Fig. 5. Competitive RT-PCR of *IL-4* and *GM-SCF* and *TNFα* in stomach tumors induced in Lewis rats (T2–5, adenocarcinomas; T1 and T6, adenomas) and WKY rats (T7–12, adenocarcinomas) by MNNG, and control WKY and Lewis stomach pyloric mucosa (negative controls) and Lewis pyloric mucosa after 14 days' MNNG exposure (positive control). The lower bands were competitors. 18S ribosomal RNA shows the relative amount of RT-cDNA.

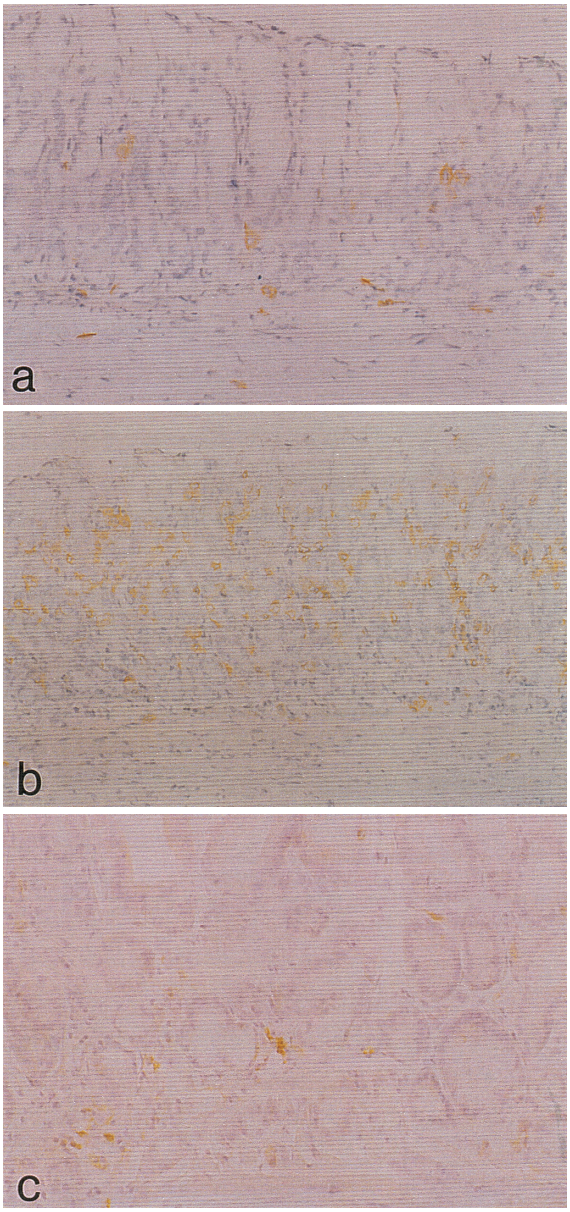

Fig. 6. Immunohistochemical staining of *integrin αE2* in the stomach tumor with *OX-62*. Brown cells are mAb-stained cells. ×200. (a) Control Lewis stomach pyloric mucosa, (b) MNNG-exposed Lewis pyloric mucosa and (c) Lewis stomach adenocarcinoma (T2).

mals; no differences were observed in ACI or Buffalo strain.

Expression of *integrin αE2* and *β7, Ii, MHC class II, B7-1, CD28* and *E-cadherin* genes in stomach tumors Having studied a range of markers which may be directly or indirectly associated with dendritic cell presence, we compared the profile of gene expression with that in MNNG-induced rat stomach tumors. Fig. 4 shows competitive RT-PCR of *integrin αE2* and *β7*, *Ii*, *MHC class II*, *B7-1* (costimulator of dendritic cells),[23] *CD28* (*B7* receptor on T cell)[24] and E-cadherin in six Lewis rat stomach tumors (T2–5, adenocarcinomas and T1 and T6, adenomas) and in six WKY rat stomach tumors (T7–12, adenocarcinomas). Control Lewis and WKY rat (8 weeks old) stomach pyloric mucosa and Lewis rat stomach pyloric mucosa after 14 days' MNNG exposure served as negative and positive controls, respectively. For *integrin αE2* and *β7*, *Ii*, *MHC class II*, *B7-1* and *CD28* genes, expression was weak or undetectable in pyloric mucosa of control animals, but it was clearly evident 14 days after MNNG exposure (positive control). In all twelve stomach tumors these genes continued to be expressed, though with different intensities and less strongly than in positive controls, especially for *integrin αE2*, *Ii*, *B7-1* and *CD28*. The results suggest that differentiated dendritic cells were present in stomach tumors, but to a lesser extent than in pyloric mucosa in the initiation stage of carcinogenesis. In contrast, the *E-cadherin* gene was similarly expressed in control pyloric mucosa, with and without MNNG exposure, and in all stomach tumors.

Competitive RT-PCR of *IL-4* and *GM-SCF* and *TNFα* in stomach tumors Fig. 5 shows that *IL-4* gene expression was not detected in stomach tumors induced in Lewis rats (T2–5, adenocarcinomas; T1 and T6, adenomas) or in WKY rats (T7–12, adenocarcinomas) by MNNG, or in control WKY and Lewis stomach pyloric mucosa. *IL-4* expression was detected in Lewis pyloric mucosa after 14 days' MNNG exposure (positive control). *GM-CSF* and *TNFα* gene expression was low in Lewis and WKY control animals, but was increased in tumors and in MNNG-exposed Lewis pyloric mucosa.

Immunohistochemical demonstration of *OX-62* and *Ii* in stomach tumors To demonstrate the presence of dendritic cells in stomach tumors, the presence of *OX-62*- and *Ii*-mAb-stained cells in stomach tumors was studied immunohistochemically. Only a few stained cells could be seen in control Lewis rat stomach pyloric mucosa (Fig. 6a for *OX-62* and Fig. 7a for *Ii*). After 14 days' MNNG exposure, increased numbers of *OX-62*- and *Ii*-mAb-stained cells were observed in the interstitial tissue of pyloric mucosa (Fig. 6b for *OX-62* and Fig. 7b for *Ii*). *Ii*-mAb-stained cells were seen in a rat stomach tumor (T2, adenocarcinoma) (Fig. 7c), but only a few *OX62*-stained cells were seen (Fig. 6c). These cells were observed in tissue of interstitial origin.

Western blot of *OX-62* An *OX-62*-stained protein band was detected in all tumors and in control pyloric mucosa by sensitive western blotting. However, no significant increase in tumors compared to control pyloric mucosa was observed (Fig. 8).

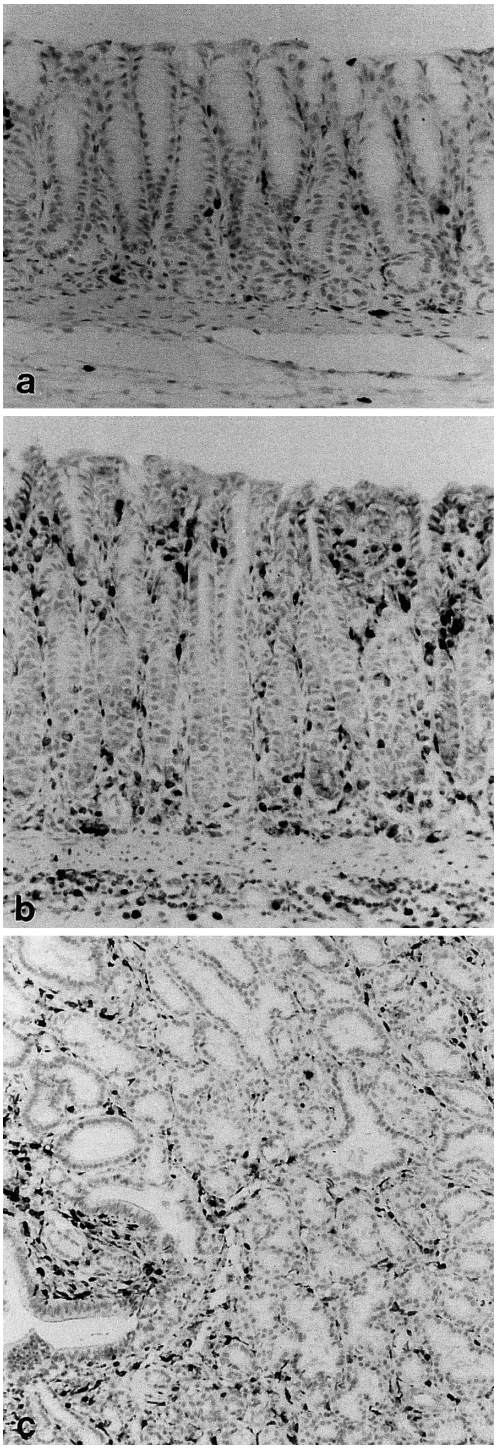

Fig. 7. Immunohistochemical staining of *Ii* in the stomach tumor with *Ii*-mAb. Dark cells are mAb-stained cells. ×200. (a) Control Lewis stomach pyloric mucosa, (b) MNNG-exposed Lewis pyloric mucosa and (c) Lewis stomach adenocarcinoma (T2).

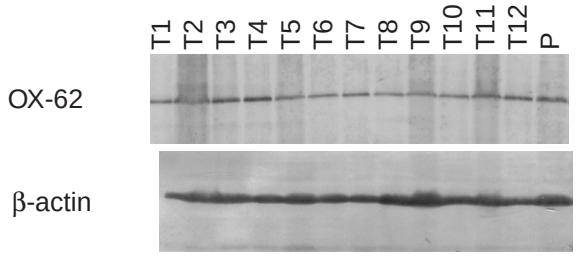

Fig. 8. Western blot of *OX-62* in the stomach tumors (T1−12) and in the control Lewis stomach pyloric mucosa (P). β-Actin shows the relative amount of protein.

DISCUSSION

The focus of our research program is the very early changes in experimental rat stomach carcinogenesis caused by MNNG.[25, 26] Previously we found that dendritic cells, which were stained with *OX-62* mAb and which expressed *MHC class I* and *II* (*MHC class II*, *Ii*) genes, *B7-1* and *B7-2*, appeared in the interstitial tissue of rat stomach pyloric mucosa after 14 days' MNNG exposure.[5, 7] In the present study we confirmed gene expression of *integrin αE2*, known to be the antigen for *OX-62* mAb,[11] and demonstrated gene expression of *integrin β7*, which is another unit of the integrin complex containing integrin αE2.[12]

At least three pathways have been proposed for the development of dendritic cells: lymphoid, myeloid and Langerhans cell (in skin) lineages.[16, 27] *IL-4*, *GM-CSF* and *TNFα* were proposed as essential cytokines for differentiation and maturation of human dendritic cells *in vitro*.[28, 29] Of these cytokines, *IL-4* and *GM-CSF* have been reported in rats.[15] At present, dendritic cell differentiation and maturation and the effects of cytokines are mainly studied with *in vitro* systems, and there are very few *in vivo* studies. In normal control animals, only a small number of dendritic cells are found in the stomach pyloric mucosa.[7] MNNG exposure for 14 days caused the appearance of dendritic cells in interstitial tissue of pyloric mucosa.[7] BrdU-labeling showed that cells, including dendritic cells or their precursors, did not proliferate in interstitial tissue of pyloric mucosa.[7] It is likely, then, that the appearance of dendritic cells in the stomach pyloric mucosa can be explained in one of two ways. First, dendritic cells may be formed by differentiation and maturation of precursor cells in the stomach pyloric mucosa under the influence of cytokines. Second, mature dendritic cells may migrate from other sources. This present study examined the first possibility.

Competitive RT-PCR demonstrated that gene expression of *IL-4*, *GM-CSF* and *TNFα* was induced by MNNG

exposure in pyloric mucosa and that the induction was several times greater in Buffalo rats than in ACI rats. Since MNNG induces a greater number of dendritic cells in interstitial tissue of stomach pyloric mucosa in resistant Buffalo rats,[7] these gene expression results suggest the possibility that dendritic cell differentiation and maturation may be induced in the pyloric mucosa from peripheral blood precursor cells by these cytokines. Dendritic cells appeared after 14 days' MNNG exposure (but not after 3 days' exposure) in rat stomach pyloric mucosa.[5] This period coincided with the time taken for *in vitro* differentiation and maturation from precursor cells. Recently, rapid recruitment of dendritic cell precursors (OX-62$^+$ MHC II$^+$) within a day from bone marrow cells was reported in rat liver[30] and after rat cardiac transplantation.[31]

We also observed gene expression of *integrin αE2* and *β7*, *MHC class II*, *Ii* and *B7-1* in MNNG-induced adenocarcinomas and adenomas, suggesting the presence of dendritic cells also in tumors. However, expression of some of these genes was less than that in MNNG-exposed Lewis rat stomach pyloric mucosa. *OX-62* immunohistochemistry and western blotting also suggested the presence of a smaller number of dendritic cells in tumors. The above results suggest that the population of dendritic cells in tumors is less than that in the pyloric mucosa after 14 days' MNNG exposure.

The presence of dendritic cells in advanced human stomach cancer has been reported, with small numbers of dendritic cells in the primary tumors being associated with reduced lymph node metastasis.[32] Lower dendritic cell presence in tumors was more frequent in lymph node metastasis-negative patients with recurrence.[33]

E-Cadherin is a cell adhesion molecule and a ligand of the integrin αE2 and β7 complex.[12] Recently it was reported that germline mutations in the *E-cadherin* gene were causes of familial gastric cancer in New Zealand.[34] Mutations in *E-cadherin* were also reported in human diffuse-type gastric carcinomas.[35] We examined changes in *E-cadherin* gene expression and protein expression in rat stomach pyloric mucosa after MNNG exposure and in 10 differentiated adenocarcinomas and 2 adenomas. However, no changes in *E-cadherin* expression were observed in this experimental stomach carcinogenesis system (data not shown).

The dendritic cell is hypothesized to be the strongest antigen-presenting cell to T helper cells,[36] the latter then commanding B cells to make an antibody for a long-term immune response. Although *CD4*, *CD28* and *IgM* expression was detected by RT-PCR and northern blot,[7] large numbers of T helper cells or B cells were not observed in interstitial tissue of pyloric mucosa examined immunohistochemically (data not shown). There remains the possibility that dendritic cells play another role in interstitial tissue of pyloric mucosa during stomach carcinogenesis caused by MNNG; further studies are required to examine this. For example, endocytotic activity of dendritic cells in rat liver[30] and cytolytic activity[37, 38] of splenic dendritic cells *in vitro* were reported.

Different susceptibility to MNNG-induced stomach cancer was reported in Buffalo and ACI rats.[8] Some genetic differences[9, 10] and some differences in gene expressions[7] were also revealed. However, further study is required to elucidate the control mechanisms of the different immune responses between the two strains.

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