Ossification of the N-Methyl-N'-Nitro-N-Nitrosoguanidine-Induced Small Intestine Adenocarcinomas in Rats

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Eighty rats out of 233 developed malignant tumors in the stomach and small intestine by administration of 100 μ g/ml N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in drinking water for 28 weeks. Fifteen lesions (30%) among the 50 small intestinal carcinomas showed ossification in the tumor, while none in the sarcomas (12 lesions) or gastric adenocarcinomas (59 lesions) showed ossification. Multifocal heterotopic bone formation was found within stroma in close approximation to the neoplastic glands. The islands of bone trabeculae were covered by osteoblast-like cells, and abundant fibroblasts in loose stroma gathered around the bony islands which enclosed osteocytes in lacunae. Neither osteoclast nor cartilage was identified. In 5 cases, ossification was extensive, which comprised the major portion of the stroma. In contrast, intraluminal calcification without ossified foci were occasionally seen in the gastric carcinoma. Ossification of the intestinal tumors correlated to the degree of mucin content ($p < 0.05$, chi square with Yates' correction), degree of neutrophilic infiltration ($p < 0.05$), and size of the tumor ($p < 0.1$). (The average size of the ossified tumor was 21.5 ± 4.0 mm, while that of nonossified tumors was 12.5 ± 1.9 mm.) The degree of tumoral necrosis, desmoplasia or depth of invasion did not seem to be related to the ossification of the tumor. The ossification rate of this experimental model was much higher than in human cases. Various histologic alterations, such as mucin leakage, inflammatory cell infiltration, necrosis and/or fibrosis, which might be caused by continuous stimulation of the strong carcinogen, may play some role in the ossification of experimental tumors.

Key Words: Ossification, Small intestine, Adenocarcinoma, MNNG, Rats

INTRODUCTION

Heterotopic ossification can occur in a variety of conditions including malignant tumors, but such an occurrence in human alimentary tract cancer is extremely unusual (Rosenbaum et al., 1984). Several cases of gastrointestinal carcinomas with ossification have been documented sporadically in humans, and it was believed that tumor necrosis and mucin in the stroma were responsible in ossification (Engel and Dockerty, 1962). Similarly, heterotopic ossification was demonstrated in experimental adenocarcinomas of the

intestine in rats (Saito et al., 1970), but they were accompanied with chondroid changes, and the details of its mechanism remain obscure. During analysis of experimentally induced gastrointestinal adenocarcinomas in rats, our attention was called to the frequent association of tumorous ossification in the small intestine-adenocarcinomas, and this prompted us to investigate the background of heterotopic ossification of gastrointestinal adenocarcinomas in an N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced experimental model.

MATERIALS AND METHODS

Using Sprague-Dawley rats, 233 rats, weighing from 150-180 g at the beginning of the experiment, received

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100 μ g/ml of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a strong carcinogen, in their drinking water for 28 weeks. The rats were allowed to chew the commercial pellet and drink water ad libitum during the whole experimental period. The animals were sacrificed 40 weeks after the beginning of the carcinogen administration, and the stomach and duodenum were separately removed. The stomach was opened along the greater curvature, pinned on a cork plate, and fixed in 10% neutral formalin. The fixed stomach and intestine were sliced into 4 or 5 longitudinal strips and were embedded in paraffin, sectioned in 4 μ m thickness, and stained with hematoxylin and eosin.

RESULT

Eighty of the 233 MNNG-administered rats developed 121 malignant tumors in the stomach and duodenum (59 gastric adenocarcinomas, 50 small intestine adenocarcinomas, and 12 sarcomas). None of the 20 control animals developed neoplastic lesions. Tumor ossification was found in 15 (30%) intestinal adenocarcinomas from 14 rats, and gastric adenocarcinoma or sarcoma were free from it (Table 1). These heterotopic bone foci were confined only within the neoplastic stroma in close approximation to the neoplastic glands (Fig. 1 and 2), where loosely textured

Table 1. Ossification of tumors in MNNG-administered rats

Type of Tumors	Nonossified	Ossified	Total
Gastric carcinoma	59	0 (0%)	59
Intestinal carcinoma	35	15 (30%)	50
Sarcoma	12	0 (0%)	12
Total	106	15 (12%)	121

Fig. 1. Histopathology of well-differentiated adenocarcinoma in the small intestine induced by MNNG (H&E, $\times 100$).

Fig. 2. Ossified stroma adjacent to the neoplastic glands. A gland is cystically dilated and contains mucinous material (H&E, $\times 100$).

fibroblasts gathered around the bony islands with several osteocytes in lacunae (Fig. 3). In contrast, calcification was encountered in both gastric and small intestine-adenocarcinomas (Table 2), and they were localized within the glandular lumen (Fig. 4). Neither osteoclasts nor chondrocytes were participated in the ossified portion. Ossification was more extensive in 5 cases, occasional in 4, and spotty in the remaining 6

Fig. 3. High-power picture of ossified stroma of small intestinal carcinoma. Osteocytes are embedded in the ossified stroma (H&E, $\times 200$).

Table 2. Occurrence of calcification in MNNG-induced adenocarcinomas

Type of Tumors	Incidence
Gastric carcinoma	9/59 (15.3%)
Intestinal carcinoma	24/50 (48.0%)
Ossified	15/15 (100%)
Nonossified	9/35 (25.7%)
Sarcomas	0/12 (0%)

Fig. 4. Calcified focus in the gastric carcinoma. Calcification is confined to the intraluminal portion and surrounding stroma is hyalinized (H&E, $\times 100$).

cases. In the former 5 cases, ossification comprised the major portion of the stroma.

The histologic features of the tumors varied from well differentiated to undifferentiated carcinomas and were accompanied by varying amounts of mucin content. Ossification was found in one-third of the well-differentiated (8/24) and moderately-differentiated (7/21), while none appeared in the poorly differentiated (0/4) and undifferentiated (0/1) carcinomas.

To draw a possible relationship between tumoral ossification and associated histopathologic findings, the amounts of neutrophilic infiltration and mucin content were compared with the presence or absence of ossification, and the statistical significance was assessed by a Chi square test with Yates' correction. Ossification of the tumor was highly associated with the degree of mucin content (Fig. 5, $p < 0.05$) and neutrophilic infiltration (Table 3, $p < 0.05$). The average size of the ossified intestinal tumor was 21.5 ± 4.0 mm (mean $\pm$ SEM), while it was 12.5 ± 1.9 mm in the nonossified tumor, but the statistical significance was equivocal (Fig. 6, $0.05 < p < 0.1$). The degree of tumoral necrosis, desmoplasia of the tumor, or depth of tumor invasion did not correlate to tumor ossification.

Table 3. Relation between neutrophilic infiltration and ossification in intestinal adenocarcinoma

Neutrophilic Infiltration	Nonossified	Ossified	Total
None or mild	20	3	23
Moderate to severe	15	12	27
Total	35	15	50

($P < 0.05$ by Yates' correction)

Fig. 5. Amount of mucin content and ossification of the tumor in the intestinal carcinoma.

Fig. 6. Size distribution of the tumors between ossified and nonossified tumor.

DISCUSSION

Heterotopic ossification in gastrointestinal carcinomas is a rare manifestation in both humans and experimental animals. Most of the reported human cases were confined to the rectum and sigmoid colon (Engel and Dockerty, 1962), and rarely described were in the stomach, appendix, prostate, gallbladder, endometrium, urinary bladder, breast, and salivary gland (Willis, 1973; Rhine and Horowitz, 1976; Randall et al. 1989).

The mechanism of tumoral ossification still remains obscure. Rosenbaum et al. (1984) suggest that gamma-carboxy-glutamic acid (GLA), a major constituent of non-collagenous bone protein osteocalcin and coagulation protein prothrombin, plays an important role in heterotopic bone formation. They also suggest that local thrombus formation with sequestration of serum protein and subsequent local recruitment of GLA and calcium precede the tumoral ossification.

In this study, neither thrombosis nor hematoma formation was found in the tumors around the ossified foci. Instead, abundant mucin with heavy neutrophilic infiltration in the mucin pool was encountered in most, as suggested in human cases (Engel and Dockerty, 1962). It has been well known that dystrophic calcification is very rare in gastrointestinal adenocarcinomas and is, if present, exclusively found in the mucinous type, although the mechanism of their association is also not yet clearly understood.

The site selectivity of tumorous ossification that occurred only in intestinal carcinomas and not in gastric carcinomas is not clear. Neuhoef (1917) describes ossification of the urinary bladder wall in dogs by transplantation of fascial tissue for its repair. He observes that ossification occurs only when the urine is acid. Abbott et al. (1938) confirm that among various epithelia they used, only transitional epithelium is responsible for inducing bone formation. Nevertheless, our results were contrary to the above observations in that ossification occurred in the intestinal tract where the lumen was alkaline, and none had ossification in the stomach where the gastric mucosa secretes acidic gastric juice.

Calcification was an additional feature in both gastric and small intestinal tumors in this study, and it was always associated with ossification in intestinal adenocarcinomas. Intraluminal localization of calcified plaques, a frequent finding in gastric adenocarcinomas, was not seen in any of the intestinal adenocarcinomas, and no correlation was found between histopathologic findings, such as the degree of mucin content, neu-

trophilic infiltration and size of the tumor, and calcification in 59 cases of gastric adenocarcinomas. There was only equivocal association between the degree of desmoplastic change and calcification ($0.05 < P < 0.1$). Even in the small intestine adenocarcinomas, calcification was noted only in the intraluminal area, while ossification was found exclusively in the stroma between the neoplastic glands. Moreover, the calcified foci were always necrotic, while ossified tissue was surrounded by osteoblast-like cells and contained viable osteocytes. The above findings imply that calcification is not homologous to ossification of small intestine adenocarcinomas and is a different process from the ossification of the small intestine carcinomas.

Ossification in experimentally-induced gastrointestinal adenocarcinoma has been documented in several studies (Szentirmay and Sugar, 1985; Saito et al., 1970). They described that mature cartilage was also seen in those cases. Although heterotopic ossification with cartilage formation is a *sine qua non* of traumatic myositis ossificans, a classical pathway of dystrophic bone formation, such ossification with chondroid element has not been described in any of the human gastrointestinal adenocarcinomas to the best of the authors' knowledge. We are not in a situation to explain why some adenocarcinomas ossify with chondroid metaplasia and others without it in animal tumors. The exact mechanism of ossification in malignancy should be clarified before the above question is answered.

In this study, 30% of small intestine adenocarcinomas showed ossification. This figure is much higher than that of human cases. Although there are several histological similarities between small intestine adenocarcinomas in humans and experimental animals, it is our experience through an MNNG-induced animal model that tumor necrosis, stromal fibrosis, inflammatory cell infiltration, and mucin leakage are more frequently seen in rat adenocarcinomas. However, it is not clear at this moment that such associated changes might be caused simultaneously by continuous stimulation of the strong carcinogen administered, or that they might be an integral feature of the animal tumor itself. Biologic differences of the tumors in humans and rats are also a possible factor, because none of the malignant lesions in this study metastasized to a regional lymph node or distant site.

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