Invasion of Cartilage by an Experimental Rat Tumor

A. R. Poole

The Research Department, The Marie Curie Memorial Foundation, Harestone Drive, Caterham, Surrey, England

SUMMARY

The in vivo invasion of cartilage of the xiphisternum by a transplantable malignant rat tumor has been investigated with histochemical methods. Staining of cartilage with toluidine blue was considerably reduced or absent at the edge of the invading tumor. Frequently, matrix staining around chondrocytes close to the tumor was much more intense than that of more remote matrix. This pericellular staining feature was also observed in cartilage from tumor-free animals treated with papain but was absent from those which received an excess of vitamin A. Appreciable intercellular ß-glucuronidase activity was observed in tumor-infiltrated cartilage, but not in normal cartilage. In chondrocytes, this enzyme was normally observed in small cytoplasmic particles (0.2 to 0.5 μ in diameter). In the presence of the established tumor, however, larger stained particles (1.0 to 2.0 μ in diameter) were frequently observed. The rate of lysosomal staining for acid phosphatase was unchanged in chondrocytes and fibroblasts of cartilage undergoing early tumor infiltration, but was increased in later cases. These results are interpreted and considered in the light of the proposal that lysosomal acid hydrolases are released from the tumor, and normal cells and their extracellular activity plays an essential role in malignant invasive growth.

INTRODUCTION

There has been much debate as to how malignant tumors invade normal tissues. This process is characterized by the destruction of normal tissue components in the vicinity of the invading tumor cells (22). It has been suggested that malignant tumors invade the tissues around them as a result of the pressure developed by tumor growth (29). Although many workers do not now consider pressure to be the main cause of tissue invasion and destruction, it may, in cases of tumors surrounded in vivo by contiguous cells, play some part in the overall destructive effect. However, in tissue culture systems, colonies of normal cells are infiltrated and destroyed by malignant cells (14) under conditions where no significant pressure could be developed. The motility of tumor cells (26) is obviously conducive toward invasiveness.

Recently, interest has arisen in the role of lysosomal acid hydrolases in malignant invasive growth. Sylven (22, 23) has demonstrated that higher concentrations of these enzymes are to be found in interstitial tumor fluids (within and at the edge of the tumor) than in normal body fluids. Much work with cartilage has also indicated that these enzymes, when appreciably released to an extracellular state, cause the destruction of cartilage matrix (3, 7).

It was noted that a transplantable malignant rat tumor, the Guerin T8 epithelioma, invaded cartilage of the xiphisternum in vivo. Hence a tumor-cartilage system for the study of invasive growth in vivo was developed. A similar in vivo model with spinal bone and the Jensen sarcoma has been previously described (25).

Cartilage lends itself to such a study for several reasons. It is weakly antigenic, and hence damage to it elicits little cellular immune response (10). Normally, a considerable inflammatory reaction, involving an accumulation of polymorphonuclear leukocytes, lymphocytes, macrophages, and mast cells, is invariably a feature of in vivo nonskeletal tissue invasion. This obviously complicates any interpretation of the process. By virtue of its organization, cartilage also permits a study to be made not only of cell damage but also of intercellular substance degradation. Histochemical methods are available for such studies, and for demonstrating acid hydrolase activities, and these have been used.

The results obtained have been examined and interpreted in the light of data pertinent to the proposal that infiltrative growth of this tumor and others is dependent upon the extracellular activities of acid hydrolase derived from normal and/or malignant cells.

MATERIALS AND METHODS

The Guerin T8 epithelioma was kindly supplied originally by Dr. L. Németh, Onkopathologiai, Kutató Intézet, Budapest, Hungary. It was transplanted into female Sprague-Dawley rats. Rats weighed approximately 175 g and were 10 to 12 weeks old when used. They were supplied by Animal Suppliers Ltd., Welwyn, England. Animals were supplied ad libitum with drinking water and Diet PRM (Dixon and Sons, Ware, Ltd., Elstree, England).

The tumor was normally maintained by transplantation to the spleen, or liver, at 4 to 6 week intervals (18). For this study, it was transplanted onto the ventral surface of the
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Xiphisternum of recipients; it was attached by a cotton ligature.

Xiphisterna from normal animals and those bearing tumors were excised in toto after quickly killing the animals by placing them in an atmosphere of nitrogen for approximately 3 min. There is no evidence to indicate that such short-term asphyxiation causes a redistribution of lysosomal enzymes, although this does occur in the livers of rats asphyxiated for 1 to 5 hr (2). Tissue was immediately frozen rapidly by plunging it into a bath of aromatic-free hexane at −68°. After 10 min, it was removed with precooled forceps and mounted on a cryostat specimen holder. Frozen sections 6 to 12 μ thick were cut within 24 hr at a cabinet temperature of −25° to −30° with a blade cooled with solid CO2. They were used immediately.

Sections were stained for 10 min with 0.5% (w/v) toluidine blue in 5% (v/v) ethanol in distilled water (15). Excess stain was removed with water until washings were colorless. Sections were then dehydrated in alcohol, cleared in xylene, and mounted in Clearmount (E. Gurr, Ltd., London, England).

Acid phosphatase was demonstrated histochemically with the Gomori method as modified by Bitensky (6), except that buffered solutions of lead nitrate-sodium-β-glycerophosphate were freed of any precipitate by filtration after incubating for only 1 hr at 37° before use (17). The rates of staining of lysosomes (5) in chondrocytes and fibroblasts were determined by measuring the time, at 5-min intervals, which they took to stain at 37° in the test medium. If lysosomes of a given cell type were only just visible after perhaps 15 min of incubation, with the majority visible at 20 min, a time of 17.5 min was recorded. Otherwise, the time at which the majority first became visible (5, 10, 15, 20, or 25 min) was recorded. Controls were prepared by incubating sections at 37° in the presence of 10 mM sodium fluoride or in the absence of added substrate. Permanent mounts were prepared as previously described.

β-Glucuronidase was demonstrated histochemically (13); the diazonium salt Fast black K (diazonium salt of 4-amino-2,5-dimethoxy-4'-nitroazobenzene, Koch-Light Laboratories, Ltd., Colnbrook, England) was used instead of diazotized pararosaniline hydrochloride. Control sections were prepared by incubating at 37° in the presence of 15 mM saccharo-1,4-lactone (Calbiochem, Los Angeles, Calif.) or in the absence of the added substrate. Incubations at 37° lasted from 5 min up to 2 hr. Sections were mounted directly in Aquamount (E. Gurr Ldt., London, England) and viewed within 2 hr.

Papain (2 times crystallized suspension in 0.05 M acetate buffer, pH 4.5, activity 13 units/mg, Koch-Light Laboratories, Ltd.) was diluted for use with distilled water and sodium chloride to give a final concentration of 0.9% (w/v) sodium chloride, 0.0125 M acetate buffer, pH 4.5, and 90 units/ml of papain. A 0.2-ml volume was injected i.p. into tumor-free rats daily at 10 a.m., 2 p.m., and 5 p.m. for a total of 5 days. At 5 p.m. on the 5th day, animals were killed by cervical fracture without receiving a further injection. Control animals were given injections in the same way with the same material, except for the omission of papain. Two animals comprised each group, and experiments were duplicated.

The palmitate of vitamin A-alcohol was obtained as a corn oil solution (1 X 10⁶ i.u./g, Sigma Chemical Co., St. Louis, Mo.). Approximately 1.5 X 10⁶ i.u./day were injected s.c. daily at 10 a.m. for 5 days. Animals were killed on the 5th day at 5 p.m. by cervical fracture. Control animals received 1.5 ml/day of Saladin oil (British Edible Oils, Ltd., London, England) injected in the same way. Groups and other experimental details were as described above for papain.

RESULTS

With respect to tumor growth, the number of days indicated refers to that number after tumor implantation. Cells of animals from which the tumor is absent (controls) are referred to as normal for convenience of description. In order to facilitate description, the cartilage plate is divided into median and lateral zones, as shown in Chart 1.

Toluidine Blue Histochemistry. Chondrocytes were clearly recognized by virtue of their morphology and the normally intensely violet-stained matrix in which they reside; at the extreme edge a reddish-pink-stained zone of matrix was observed, adjacent to which was the pannus of normal cartilage composed of fibroblasts.

The tumor cells grew toward the cartilage and also grew along its edge (2 to 3 days). When fibroblasts occupied a position intermediate between the tumor cells and the cartilage, changes in matrix staining were not detected (Fig.
The tumor cell population in the vicinity of the cartilage increased in density, and at 7 days tumor cells were often present in lateral zones (Fig. 2) on dorsal, as well as ventral, surfaces of the cartilage. Wherever a population of these malignant cells was present in areas directly adjacent to the cartilage, there was less intense matrix staining of a reddish-pink color (Fig. 4). At 7 days, tumor cells were infiltrating the cartilage, in parts eroding it away. There was little or no matrix staining at the invading edge (Fig. 4), and an overall decrease in staining density was recorded throughout the cartilage (compare Figs. 1 and 2), central areas assuming a reddish-violet color. Usually, the invasion edge was well defined, but sometimes, after 14 days, chondrocytes and their extracellular matrix were observed in the tumor remote from the invasion edge. By this time, tumor cells usually completely covered both surfaces of the cartilage.

It was common to observe irregularities of chondrocyte morphology in areas adjacent to tumor cells (Figs. 4 and 5). Chondrocytes more remote, even those in areas of pink matrix staining, appeared quite intact (Figs. 4 and 5). Areas of gross tumor necrosis were sometimes noted close to the cartilage (1-mm distance), but never directly applied to it. However, matrix staining was not found to be further reduced close to such necrotic areas.

Frequently, pericellular haloes of reddish-pink matrix staining were associated with chondrocytes close to tumor cells, the staining of matrix more remote from the chondrocytes being much less intense (Figs. 5 and 6).

Free-living normal cells, such as polymorphonuclear leukocytes, lymphocytes, and macrophages, were not usually observed at the invading edge of the tumor. Individual mast cells were occasionally seen, but exhibited little sign of degranulation.

Experiments were also conducted to study the in vivo effects of papain and vitamin A on matrix staining. By reference to the control animals, it was apparent that injection of either vitamin A or papain caused an overall reduction in the intensity of matrix staining, producing a central red-violet color and a more marked pale pink staining at the edge of the cartilage, of the kind observed adjacent to the infiltrating tumor. In the papain-treated animals, the aforementioned more intense pericellular staining around chondrocytes was frequently observed (Fig. 7) at the edge of the cartilage. These staining haloes were not usually seen in cartilage of vitamin A-treated animals (Fig. 9).

β-Glucuronidase Histochemistry. Sections of normal and tumor-invaded cartilage, 12 μ thick, incubated for 30 min in the test medium exhibited a purple-black cytoplasmic staining of chondrocytes. In control sections incubated at 37° in the absence of substrate, or in the presence of saccharo-1,4-lactone, chondrocytes stained a faint light brown.

In the normal chondrocytes (no tumor present), staining after 30 min at 37° in the test medium was cytoplasmic and of both a diffuse and a particulate nature. The stained particles were usually spherical and 0.2 to 0.5 μ in diameter, although some cells contained 1 or 2 larger stained particles (not perfectly rounded) 0.8 to 1.0 μ in diameter. Intercellular matrix staining was only slight, if observed (Fig. 10). Fibroblast staining was of the kind observed in chondrocytes, except that the larger particles were not observed.

Cartilage undergoing infiltration stained differently in several respects. In a zone directly adjacent to the tumor, chondrocytes were either poorly stained (mainly diffuse) or unstained (Fig. 8). This was particularly evident with the 14-day tumors. Chondrocytes more remote from the tumor at this stage possessed many stained cytoplasmic particles. However, the larger particle type was observed more frequently (3 to 5 per cell) and was 1 to 2 μ in diameter (Fig. 11). The staining of fibroblasts, where present, was apparently unchanged from that of normal fibroblasts.

After 30-min and 60-min incubations in test solutions at 37°, tumor-infiltrated cartilage frequently exhibited marked matrix staining, often intense (Fig. 8), in areas which stained reddish-violet with toluidine blue. This density of staining was usually greater in areas remote from the chondrocytes.

Acid Phosphatase Histochemistry. After incubations in the test solution at 37°, both normal fibroblasts and chondrocytes exhibited both a diffuse and a particulate (lysosomal) cytoplasmic staining of the kind observed for β-glucuronidase. The stained particles were 0.2 to 0.5 μ in diameter. Cells of control sections were unstained. In the presence of the tumor, normal cells which stained appeared to contain comparable numbers of lysosomes.

The rates of staining of lysosomes in chondrocytes and fibroblasts in the presence and absence of the tumor were determined at 7 and 14 days. The results are shown in Table 1. At 7 days, no significant differences were detected in tumor-invaded cartilage in either cell type. However, at 14 days, rates were significantly increased in both cell types.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Normal xiphisternum</th>
<th>Xiphisternum + 7-day tumor</th>
<th>Xiphisternum + 14-day tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chondrocytes</td>
<td>21.5 ± 2.5 (5)</td>
<td>16.5 ± 3.7 (5)</td>
<td>11.6 ± 1.2 (3)</td>
</tr>
<tr>
<td>Fibroblasts of pannus</td>
<td>21.9 ± 4.1 (4)</td>
<td>14.2 ± 4.2 (3)</td>
<td>15.0 ± 1.8 (4)</td>
</tr>
</tbody>
</table>

DISCUSSION

A reduction in the intensity of metachromatic staining of cartilage matrix with toluidine blue (15), accompanied by a color change from violet through red-violet to pink and finally colorless (A. R. Poole, to be published), is associated with a loss of mucopolysaccharide and protein (15). Hence,
the changes observed during the growth of the tumor in contact with cartilage reflect a tumor-induced matrix degradation.

Matrix degradation in vivo is apparently normally caused by the activity of lysosomal acid hydrolases (3, 7), in particular cathepsin D (28). A purified lysosomal preparation isolated from the Guerin T8 tumor will readily degrade in vitro the matrix of xiphoid cartilage (A. R. Poole, to be published). It is thus significant that β-glucuronidase was present in the matrix ahead of the invading tumor. It is normally present in this tumor (18) and in other tissues (13) in lysosomal and/or extralysosomal cytoplasmic sites. It is unlikely that this matrix staining is an artifact caused by enzyme which has leaked out of chondrocytes after isolation of the tissue from the animal, since the enzyme was generally more concentrated in zones remote from the chondrocytes of infiltrated cartilage. The extracellular presence of other lysosomal enzymes in vivo is suggested by these findings. Lysosomal leucine naphthylamidase activity, which is nonproteolytic (4), has also been detected histochemically in an extracellular state at the edges of invading tumors (24).

Since the degradative effect of the tumor on the matrix was detected only when intimate contact existed between the two, and only infrequently were lymphocytes, polymorphonuclear leukocytes, macrophages, or mast cells seen at this interface, the tumor would appear to be capable of degrading the cartilage directly.

A direct degradative effect of tumor cells on the matrix not involving chondrocytes was indicated by the haloes of more intense pericellular staining which were frequently found around chondrocytes a little remote from the tumor (see also Ref. 21). These haloes clearly reflect either less matrix degradation around chondrocytes or, more likely, a compensatory synthesis of matrix by them. This would be expected if the enzymes digesting the pericellular matrix originated from a remote cellular source. In this respect, it was notable that there was a striking resemblance between the degradative effects of the tumor and papain, which is an enzyme capable of direct matrix degradation (16), whereas with excess vitamin A, which induces a leakage of lysosomal enzymes from chondrocytes (9), such haloes were infrequently observed.

Noninvolvement of chondrocytes and fibroblasts at 7 days was also indicated by Bitensky's lysosomal acid phosphatase fragility test (5). Increased lysosomal staining rates, which were not detected at this stage, have demonstrated early disturbances in lysosomal membrane permeability to exogenous substrate associated with an extracellular release of lysosomal enzymes (1, 19). At 14 days, however, increased staining rates indicated a possible release of lysosomal enzyme from the normal cell populations. Some cell lysis at this stage was also indicated at the invading edge by a loss of cellular β-glucuronidase activity. Disturbances in cellular metabolism were also suggested at 14 days by the occurrence in chondrocytes of increased numbers of the larger stained cytoplasmic particles, which probably represent autophagic vacuoles/cytolysomes (17). These are more commonly found in damaged cells (20). Glauert et al. (11) have also often found such large vacuoles in chondrocytes releasing lysosomal enzymes (8). Their attractive proposal that a preliminary extracellular digestion of matrix components may be followed by an intracellular digestion of phagocytosed partly digested material in these vacuoles deserves consideration in this case. Such vacuoles are found in starved cells (20), and, since the tumor has usually almost entirely encapsulated the xiphisternum at 14 days, starvation could also be a contributory factor to cell damage at this stage.

Lysosomal enzymes, which normally have acid pH optima, should be capable of functioning together in the extracellular environment at the edges of invasive tumors. A purified lysosomal preparation isolated from the Guerin T8 tumor can enzymically degrade xiphoid matrix in vitro at a bulk phase pH of 6.0 and 7.0 (A. R. Poole, to be published). Increased amounts of interstitial lactic acid at tumor peripheries (12) would tend to lower pH. Also a pH of up to 2 units lower than that of the bulk phase probably exists in the pericellular zone (26).

In addition to mediating normal tissue destruction at the edge of the tumor, these extracellular enzymes also appear to be capable of facilitating the detachment of tumor cells from the solid tumors (27), which is conducive toward metastasis formation. Hence, there is good reason that their role in malignant growth should be further studied.

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