ABSTRACT

Naturally occurring renal adenocarcinoma in North American leopard frogs, *Rana pipiens*, metastasize frequently (77%) when these ectothermic animals are kept in a warm environment but not when they are kept cold. We have found that explants of these tumors secrete collagenase, an enzyme capable of dissolving connective tissue fibers and found previously to be closely correlated with metastatic colony-forming capability of murine mammary tumors, and that the amount released sequentially rises and falls as the ambient temperature is shifted between metastasis-permissive and -inhibitory levels. In contrast, normal frog renal tissue has low collagenase output, unaffected by temperature changes.

INTRODUCTION

Some populations of wild outbred North American leopard frogs, *Rana pipiens*, are afflicted with a herpesvirus-transmitted renal adenocarcinoma otherwise known as the Lucke carcinoma (8, 10, 11). Prevalence of these tumors has become reduced in recent years (14, 15). Some tumor-bearing individuals have secondary tumor deposits in other organs (12), and the incidence and degree of metastatic spread are reported to be greater in animals subject to elevated ambient temperature (9). The tumor occurs in a poikilothermic vertebrate with a body temperature that closely follows the environment. It is, therefore, possible to manipulate the behavior of these tumors both in vivo and in vitro, as a means of identifying properties which associate exclusively with metastasis-permissive and -inhibitory conditions.

Initial experiments confirmed that living Lucke carcinoma cells labeled with fluorescein isothiocyanate or with Selenomethionine and inoculated into cold-adapted (4°) frogs reach all organs rapidly and that there are no significant quantitative differences from the findings in warm animals at comparable times after inoculation. The fluorescein isothiocyanate labeling method provides direct visual evidence of the presence of these cells in frozen sections of organs examined in a UV microscope (18). The effects of temperature on metastatic spread in this animal are, therefore, not due to incidental differences in tumor cell dissemination secondary to changes induced in blood viscosity and flow pattern. It follows that, in this tumor system, it may be possible to gain insight into some fundamental mechanisms underlying metastasis by altering a single physical variable.

In the current investigation, the unique opportunities afforded by the Lucke carcinoma to study metastasis-associated properties have been used to evaluate the role of the enzyme collagenase in metastatic spread. Earlier morphological studies on invading neoplasms and their metastases (20, 21) had implicated collagenolytic activity in tumor dissemination and colony formation. These showed extensive destruction of epithelial basement membranes and of fibrous (type I) collagen at the invasion front in primary tumors and at the boundary between carcinoma cells and surrounding stroma in metastases. Following this, biochemical investigations confirmed the presence of substantial quantities of the neutral metalloprotease collagenase in carcinomas of various organs in humans (9) and other vertebrates (22, 27). Further evidence that collagenase is functionally involved in tumor spread was provided by the observation that spontaneous mammary tumors that have high pulmonary colonization potential have significantly (p < 0.001) greater output of collagenase in vitro than those with low pulmonary colonization potential (22). It was, therefore, decided to examine whether the Lucke carcinoma secretes collagenase and, if so, whether increased output by the neoplasm is specifically associated with metastasis-permissive conditions. A brief abstract describing some of this work appeared earlier (17).

MATERIALS AND METHODS

Spontaneous Lucke adenocarcinomas were aseptically excised from the frogs in which they were found, and weighed portions of the tumors were finely minced in Wolf-Quimby amphibian culture medium. Normal frog kidneys (i.e., contralateral unaffected organs of tumor-bearing animals as well as kidneys from non-tumor bearers) served as controls and were cultured under the same conditions as tumors. Tissue fragments were established in explant culture in 25-sq cm flasks with 2 ml of Wolf-Quimby medium containing 10% newborn calf serum for 2 to 3 days at room temperature and subsequently maintained in serum-free medium, while successively exposed to a series of high and low temperatures for intervals of 2 to 4 days. The design of the experiment was to expose each set of explants to a cycle of temperatures which are metastasis-permissive (30°) or inhibitory (20° down to 7°) in vivo (9) and to assay whether there are phase-related, reversible shifts in collagenase output.

Accordingly, supernatants were harvested from each culture every 1 to 3 days, stored, and assayed catalytically for collagenase as described by Tarin et al. (22). Briefly, this consisted of measuring released soluble radioactivity from type I collagen substrate (24) prepared from rat tail tendon (22) and labeled by acetylation with [14C]acetic anhydride (2). The activities in the supernatants were expressed as units of collagenase/mg wet weight/day, where 1 unit represents the amount of enzyme required for degradation of 1 μg of collagen during 16 hr at 25°. Tissue mass was quantitated by wet weight, because DNA and protein assays destroy a tissue sample and are, therefore, incompatible with serial observations of synthetic activity. Moreover, we have found that the specific DNA content (μg DNA/mg wet weight) of the Lucke tumor falls

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within the range of values for normal kidneys. The cell contents/unit weight of tumor and normal tissue explants are, therefore, comparable.

The viability of all cultures was checked by measurement of glucose uptake (6) and by microscopy. Lactate accumulation in culture fluids, resulting from anaerobic metabolism of exogenous glucose, was measured indirectly using the lactate dehydrogenase reaction. DNA content was estimated (1) after disruption of frozen tissue with a dismembrator (Braun, West Germany).

Statistical comparisons were made with the single-tailed Student t test.

RESULTS AND DISCUSSION

Six of the 7 Lucke tumors studied released an enzyme which degraded type I collagen at pH 7.4 at 25°. Its activity was totally inhibited by EDTA and newborn calf serum but not by inhibitors of serine proteases, such as phenylmethylsulfonyl fluoride and soybean trypsin inhibitor. The enzyme released into the supernatant was, therefore, a neutral metalloprotease which was found to cleave the collagen substrate in a specific site, liberating the characteristic reaction products, demonstrable by polyacrylamide gel electrophoresis (Fig. 1), of a true vertebrate collagenase (5). In all samples, most of this collagenase was in a latent form requiring trypsin activation prior to measurement (22). The single tumor which did not produce collagenase was taken from a cold-adapted animal and is discussed further below.

In addition, supernatants from both of 2 Lucke carcinomas tested showed capability of cleaving pepsin-extracted placental type IV collagen (19). This collagenolysis was increased by trypsin activation of the supernatant and totally inhibited by EDTA but not by phenylmethylsulfonyl fluoride or soybean trypsin inhibitor. Quantitative studies on release of this enzyme (type IV collagenase) by explants at various temperatures are in progress and will be published separately. The account of quantitative, temperature-dependent aspects of collagenase secretion, which follows below, refers entirely to the enzyme capable of digesting type I (fibrous) collagen (collagenase type I).

The findings in all 6 tumors found to produce collagenase were substantially similar and are, therefore, presented collectively, except where indicated. During initial culture at room temperature (20–25°), explants from normal frog kidney and from Lucke carcinomas released low levels of collagenase (see Table 1 and Charts 1 and 2). When the incubation temperature was increased to 30°, the mean output of this enzyme from normal kidney explants was not significantly affected (p > 0.1), while that from tumor explants was substantially and significantly (p < 0.001) increased (Chart 1). Returning the tumor explants to room temperature resulted in a significant (p < 0.02) fall in collagenase output for 3 of the tumors. The remaining 3 continued to secrete collagenase at the same average rate as when they were being cultured at 30°. However, when cultured explants from one of the latter were transferred to a temperature-controlled cabinet at 7°, a fall in the enzyme output to the level produced before the temperature was raised was observed. After 2 to 4 days at 7°, the same explants were passed through a further 30°–7° cycle, and they produced a second and larger peak of collagenase, confirming that the original fall in enzyme activity on first lowering the temperature to 7° was not due to death of the tissue (Chart 2). We infer that, with the remaining 2 tumors that...
did not show a fall in collagenase on returning the explants to room temperature, we did not lower the temperature sufficiently to trip the switch which turns off raised production of this enzyme.

Our observations show that glucose consumption, by both tumors and normal renal tissue, rose concomitantly with increase in incubation temperature and thus, for tumors, paralleled rise in collagenase output. It could, therefore, be argued that elevated enzyme production was merely a reflection of increased metabolic rate. There are several lines of evidence which refute this supposition. The most powerful is that normal renal tissue did not produce more collagenase, although its glucose consumption rose to the same extent as that of tumor tissue when the temperature was raised (Chart 1). Measurement of lactate production by tumor and by normal kidney explants confirmed that there is no further hidden rise in metabolic rate due to a major shift in the pattern of glycolytic metabolism in tissue from either source (data not shown). Moreover, for 3 of the tumors, elevated collagenase release was maintained on return to room temperature in the face of falling glucose consumption and is, therefore, not directly linked to this aspect of metabolism.

Our findings demonstrate (a) that Lucke renal adenocarcinomas predictably liberate elevated quantities of collagenase when exposed to raised environmental temperatures known to provoke metastasis in vivo, (b) that this characteristic is reversible on subsequently lowering the temperature to metastasis-inhibitory levels, and (c) that temperature-induced changes in enzyme release are not just a secondary consequence of altered metabolic rate nor of a shift in the glycolytic pathway in tumor tissue relative to normal kidney.

The 6 tumors which all showed these properties were a heterogeneous group: one arose in a pronephric rudiment in a tadpole and another in a triploid frog of laboratory origin (16). Tissue for the latter sample had been maintained by successive transplantation in the anterior eye chamber. The remainder were spontaneous neoplasms arising in a captive colony of frogs. The tumor which did not express temperature-dependent collagenase production was derived from a cold-adapted frog (i.e., one kept chilled for 3.5 months in the refrigerator) in which the herpesvirus is known to be in a replicative phase (13). A further relevant observation was that disaggregated cells from one of the tumors consistently failed to produce collagenase throughout the culture period, even though explants (in which some tissue organization is retained) from the same tumor released substantial amounts of this enzyme. The ratio of glucose utilization in these cell cultures to that in the explant cultures of the same tumor was such that collagenase output should easily have been detectable.

The demonstration, that collagenase release by Lucke tumor explants in vitro is strongly correlated with conditions which encourage metastasis in vivo, provides evidence that the enzyme is a causal element in the process of metastatic spread. The release of collagenase provides a plausible mechanism for the extensive destruction of interstitial fibrous (type I) collagen seen microscopically at the boundary between invading tumor cells and adjacent stroma. Epithelial and vascular basement membranes are also lysed as the tumor advances, and the presence of a specific metalloprotease against collagen type IV in this and other (7) neoplasms could be contributory to invasion and metastasis. The degree of destruction (see above) of type I collagen is orders of magnitude greater than that of collagen type IV, but it remains possible that both degradative processes are rate-limiting events in metastatic spread. This concurs with conclusions based on previous observations in this laboratory on murine mammary tumors (22), where it was found that those capable of heavy pulmonary colonization after vascular dissemination secreted substantially more collagenase (type I) than did noncolonizers. However, collagenase is not a protein uniquely produced by neoplasms and can be detected in many normal tissues, elevated levels commonly being observed in circumstances such as remodeling of bone (25), wound healing (3), and amphibian tail resorption (4). In addition, high collagenase activity is seen in certain nonneoplastic, inflammatory conditions, such as rheumatoid arthritis (26), characterized by marked destruction of collagenous elements of the interstitial connective tissue. Its presence in nonneoplastic situations is, therefore, associated with either accelerated collagen turnover or circumstances in which breakdown of the former elements of the connective tissue exceeds synthesis. Consequently, we provisionally interpret the high output of collagenase which accompanies the inexorable connective tissue destruction seen in invading tumors and metastatic colonies (20, 21) as indicative of disordered regulation of production or activity of this enzyme. Strictly, the mode of assay used measures collagenase activity rather than enzyme protein, and the observations could conceivably reflect differences in thermolability of normal and neoplastic amphibian collagenases. Nevertheless, this does not affect the functional implication that tumors have greater collagenolytic capability than does normal tissue at elevated, metastasis-permissive temperature.

The cells of one of the tumors studied in this investigation have also been shown to be capable of metastatic deposit formation in animals kept at 30° after intravascular inoculation (23), and this provides direct evidence that cells from one of the tumors we studied in vitro are actually capable of colonization in vivo. These findings show that it is now technically feasible to use temperature control as a probe in further studies on this tumor to seek understanding of the genetic regulation which might account for the metastatic phenotype.

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REFERENCES

Frog Renal Adenocarcinoma Collagenase


Fig. 1. Polyacrylamide gel electrophoresis of reaction products of collagenase assays, demonstrating specific cleavage of type I collagen by Lucke tumor collagenase. Molecular weight markers were: rat tail type I collagen (M, 190,000) and α chains (M, 95,000), bovine serum albumin (M, 67,000), ovalbumin (M, 45,000), and myoglobin (M, 17,000) (Lane 7); 8 μg rat tail type I collagen (Lane 2); as Lane 2 incubated with trypsin-activated Lucke tumor culture supernatant (Lane 3); as Lane 3 plus 20 mM EDTA (Lane 4); as Lane 3 plus 0.3 mM phenylmethylsulfonyl fluoride (Lane 5); and as Lane 3 plus 1% newborn calf serum (Lane 6). The density of the collagen substrate bands in Lanes 3 and 5 is reduced concomitantly with the appearance of specific reaction products.
Temperature-dependent Elaboration of Collagenase by the Renal Adenocarcinoma of the Leopard Frog, *Rana pipiens*

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