Secretion of Type IV Collagenolytic Protease and Metastatic Phenotype: Induction by Transfection with c-Ha-ras but not c-Ha-ras plus Ad2-E1a

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ABSTRACT

Activated ras oncogene transfection into suitable recipient cells has been shown to induce the metastatic phenotype (Thorgeirsson, et al., Mol. Cell. Biol., 5: 259-262, 1985). We have used this model system to study the correlation of basement membrane collagenolysis with metastatic propensity. The c-Ha-ras oncogene alone, or combined with v-myc, transfected into early passage rat embryo fibroblasts, induce these cells to secrete high levels of type IV collagenolytic metalloproteinase and to concomitantly exhibit a high incidence of spontaneous metastases in nude mice. Cotransfection of c-Ha-ras plus the adenovirus type 2 E1a gene yields cells which are highly tumorigenic but nonmetastatic and fail to produce type IV collagenase. This effect is due to a suppression of collagenase elaboration, not increased production of a collagenase inhibitor, and not decreased production of a collagenase activator. The characteristics of the collagenase are identical to tumor type IV collagenase described previously. The nonmetastatic cells which failed to produce type IV collagenase retain the ability to secrete high levels of plasminogen activator. Transfection with the protooncogenic forms of Ha-ras or mos, or spontaneous transformation of NIH 3T3 cells or chemical transformation of BALB 3T3 cells yields cells which fail to produce collagenase, are tumorigenic, but totally nonmetastatic. These data support a biochemical linkage of type IV collagenase expression with the metastatic phenotype in this rodent system.

INTRODUCTION

A metastatic colony is the end result of a complex series of steps involving multiple gene products. It is likely that in some cases the augmented metastatic potential of certain tumor cells may be due to increased expression of specific gene products which confer a selective advantage. Transfection of the c-Ha-ras oncogene into suitable recipient cells (1-4) constitutes a powerful experimental model to identify putative gene products augmented in highly metastatic tumor cells compared to their nonmetastatic counterparts. In the present study, we have used this model to study the correlation of metastatic propensity with secretion of basement membrane collagenase.

Various forms of the ras oncogene family are capable of transforming a variety of cell lines (3-5). In addition to producing tumors, ras family-transformed cells also express the metastatic phenotype in nude mice (1). Either the cellular or viral form of the Harvey ras oncogene will induce murine 3T3 cells, adult rodent fibroblasts, and diploid rat embryo fibroblasts to produce metastases following i.v. (lung colonization) or s.c. injection (spontaneous metastases). In our previous studies this induction of metastases was not due to a reduction in the susceptibility of these cells to natural killer cell or macrophage killing (1). Spontaneously transformed 3T3 cells, or 3T3 cells transfected with the ras protooncogene are highly tumorigenic but fail to produce any metastases. Furthermore, if diploid rat embryo fibroblasts are cotransfected with the ras oncogene plus the adenovirus type 2 E1a oncogene, this results in a virtual complete suppression of the metastatic phenotype (6). The suppression is not due to an alteration in the level of ras transcript (6). The ras versus ras plus E1a transfectants offer a unique means to study specific cellular factors which may be regulated in parallel with the metastatic phenotype.

We have focused our attention on one of the steps critical for metastatic progression: the degradation of basement membrane (type IV) collagen. Type IV collagen is the structural scaffolding of the basement membrane, a continuous laminar matrix separating tissue compartments and surrounding blood vessels, muscles, and nerves. Tumor cells must be able to traverse this connective tissue barrier in order to metastasize. Our previous studies using established cell lines or freshly isolated tumor cells demonstrated a correlation between metastatic potential and the ability to degrade basement membrane collagen (7). However, some of the tumor cell lines used were not cloned, and not all lines were derived directly from a common parent. The ras transfection system offers the advantage that all transfectants are cloned and derived from a common parent population. The ras versus ras plus E1a-transfected rat embryo fibroblasts exhibit a very stable differential metastatic phenotype. The aim of the present study was to correlate the collagenolytic activity secreted into the media of these transfected cells with their metastatic propensity in nude mice.

MATERIALS AND METHODS

Cell Lines. Second passage REF1 were obtained from Flow Laboratories, Inc. NIH/3T3 cells and REF were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 100 μ units/ml penicillin, and 100 μg/ml streptomycin. When subconfluent they were passaged at a 1:5 ratio.

The mouse embryo cell line BALB/3T3 clone A31-1-1c at passage 9 was used to produce eight derivative cell lines obtained from type III transformed foci induced with different chemical carcinogens or by spontaneous transformation. All these cell lines were cultured in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Morphological transformation was induced by plating 1 x 10⁵ cells in 60-mm dishes and, 18 h later, exposing them to the carcinogen in complete medium. The cultures were then washed, fed twice weekly for 5 weeks, and scored for type III transformed foci. Some foci were removed by scraping, and expanded and passaged in the same medium. These transformants were induced by 72-h exposures to one of the following agents: 2 x 10⁻¹⁷ M benz[a] pyrene, 3 x 10⁻⁴ M sodium arsenite, 6 x 10⁻⁶ M cadmium chloride, and 6 x 10⁻⁷ M potassium chromate; and by 30-min exposures to N-methyl-N'-nitro-N-nitrosoguanidine, 0.5 μg/ml (F2) or 1.0 μg/ml (F3, F5). The culture and transformation methods were described (8, 9).

DNA Transfection. Transformed rat embryo cell lines, obtained by

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2 The abbreviations used are: REF, rat embryo fibroblast; PA, plasminogen activator.
transfection of various oncogenes into second passage rat embryo cells, have been previously described (6). Cell lines 1 R, 2 R, 3 R, 4 R, and 5 R were derived by transfection of the plasmid pEJ, which contains the activated form of the c-Ha-ras oncogene as cloned from the bladder carcinoma cell line T24. Cell lines designated with the prefix "R" were derived by cotransfection of the plasmids pEJ and pEla. pEla contains 2.5 kilobases of DNA sequences from the left end of the adenovirus type 2 genome, and encodes the E1a 12S and 13S transcripts. Detailed descriptions of these oncogene-containing plasmids, verification of the expression of the transfected oncogenes in the transformed cell lines, and the methodologies of DNA transfection have been described (6).

Transformed rat embryo cell lines were transfected with c-mos [transforming clone pT64 developed by T. Wood et al. (11) and kindly given by G. Van de Woude], proto-Ha-ras or N-ras oncogenes as already described (1, 4).

Metastases Assay. Cells were cultured and harvested as described (1, 4, 6). For experimental metastases, a 0.1-ml portion (5 x 10^4 cells) was injected into the lateral tail vein of 4- to 5-week-old female athymic nude mice. For spontaneous metastases 5 x 10^5 cells in 0.2 ml were injected s.c. into the right posterior flank or the subcapsular region. Mice given injections i.v. were sacrificed 3 weeks after injection; mice given injections s.c. were sacrificed after 2 months. Metastases were identified and histologically verified as described (4, 6).

Protease Assay. Type IV collagenase activity and plasminogen activation activity was measured in serum-free conditioned media (24 h of incubation of 5 x 10^5 subconfluent cells) using methods described previously (12-16). The classes of transfectants studied here showed no differences in their viability following 24-h growth in serum-free media.

RESULTS

Transfection of the ras oncogene into NIH/3T3 cells resulted in stable clones which were highly tumorigenic and also produced metastases in nude mice. Injection of 1 x 10^5 cells into the tail vein resulted in the formation of greater than 200 lung colonies in 10 of 10 mice given injections. In confirmation of our previous report (1), the ras transfected NIH/3T3 cells also produced increased amounts of type IV collagenase (Fig. 1), compared to the NIH/3T3 untreated cells. In contrast, transfection with the protooncogene form of mos (transforming clone pT64) or Ha-ras linked to a promoter (4) resulted in tumorigenic but nonmetastatic cells which also failed to produce significant amounts of type IV collagenolytic activity.

Spontaneously transformed NIH/3T3 cells were previously shown to be tumorigenic but nonmetastatic and to not produce type IV collagenase (1). In order to extend this control profile to other forms of transformation, we studied a series of BALB/3T3 cells transformed either spontaneously or by chemical carcinogens. Treatment with N-methyl-N'-nitro-N-nitrosoguanidine, potassium chromate, cadmium chloride, sodium arsenite, or benzo[a]pyrene produced malignant transformation, and the cloned lines derived from type III foci were tumorigenic in 5 or 6 of 6 nude mice given injections s.c. of 5 x 10^5 cells, but none of the mice bearing s.c. tumors exhibited metastases after 2 to 3 months. Spontaneously transformed BALB/3T3 cells and all the chemically transformed lines (all of which were tumorigenic) failed to produce significant amounts of type IV collagenase (Fig. 2).

Fig. 1. Type IV collagenolytic activity produced by NIH/3T3 cells transfected with oncogenes. The collagenolytic activity is expressed as the ng of type IV collagen substrate degraded per million cells (16 h). The mean of four separate determinations is shown; bars, SD. Metastases are expressed as the mean number of metastases produced in the lungs of 10 nude mice given injections i.v. of 5 x 10^5 cells. The c-mos and the proto-Ha-ras oncogene transfectants were highly tumorigenic but failed to produce metastases.

Fig. 2. Type IV collagenolytic activity produced by BALB/3T3 cells transfected by chemical carcinogens. The collagenolytic activity is expressed using the same units as in Fig. 1. The transformation was induced by N-methyl-N'-nitro-N-nitrosoguanidine, cadmium chloride, sodium arsenite, potassium chromate, benzo[a]pyrene, as reported in "Materials and Methods." All transfectants were tumorigenic in nude mice (6 of 6) but failed to produce any metastases after 3 months. All transfectants failed to secrete significant amounts of type IV collagenolytic activity.
even though they were highly tumorigenic. The in vivo growth rate of the tumors produced by ras plus E1a versus ras transfec-
tants was identical, 5 x 10^6 cells yielding 1-cm tumors within
2 weeks. The ras plus E1a transfecants produced virtually no
metastases after i.v. injection (Fig. 3). The type IV collagenase
production by all of the transfected rat embryo fibroblasts
consistently paralleled their metastatic behavior, without excep-
tion. The ras plus E1a transfectants failed to produce significant
amounts of type IV collagenolytic activity (active protease plus
total metalloprotease activity susceptible to trypsin activation)
comparable to the primary rat embryo fibroblast controls (Fig.
3). The metastatic ras-transfected cells produced up to 30 times
more total collagenase compared to the nonmetastatic ras plus
E1a-transfected cells. The differential secretion of collagenase
by the series of transfected rat embryo fibroblasts (Fig. 3) was
verified by gel electrophoresis of the digestion products (data
not shown), as well as by conditioned media dilution and
digestion time course studies. Example data is shown in Fig. 4.
The collagenolytic activity produced by as little as 8 x 10^6
metastatic cells (~R or Ren series: Fig. 4A, dilution 4) was still
detectable, whereas the activity produced by 10^7 nonmetastatic
double transfectants (RE series) was insignificant (Fig. 4A,
dilution 1), even after a 16-h incubation period (Fig. 4B).
The type IV collagenolytic activity produced by the ras alone
or by ras plus myc-transfected rat embryo fibroblasts was char-
acterized to study its similarity to the type IV collagenase
produced by murine or human cells (12, 15). Thirteen (Fig. 4A,
line 4R) to 30% (Fig. 4A, line 3.7) of the secreted collagenase
was active. The rest of the activity required trypsin activation.
All of the latent collagenase activity was inhibited by 20 mM
EDTA, but not by 5 mM N-ethylmaleimide and 5 mM phenyl-
methanesulfonyl fluoride. All of the collagenolytic activity was
retained on the Mono-Q anion affinity column and eluted with
0.185 M NaCl as a single major peak (Fig. 5). The type IV
collagen digestion products on gel electrophoresis were consist-
ent with a single major cleavage 25% of the distance from the
amino terminal end of the type IV collagen substrate, as de-
scribed previously (14, 15). Thus, the collagenolytic activity
secreted by the metastatic transfected rat embryo fibroblasts is apparently identical to the tumor type IV collagenolytic activity described previously (12).

The difference in secreted collagenolytic activity between the ras and the ras plus E1a cell lines could be due to the presence or absence of secreted protease inhibitors or protease activators. In order to test this possibility, we analyzed the effect of the media conditioned by ras plus E1a transfectants on the collagenolytic activity present in the media conditioned by ras transfected cells. Conditioned media from the two counterpart cells were mixed in equal amounts and concentration, with or without individual activation with trypsin. No inhibition of the type IV collagenolytic activity produced by ras plus v-myc transfected REF (3.7) combined with conditioned medium from ras plus E1a transfected REF (RE2). Units are expressed as in Fig. 1A, medium of 3.7 alone, without trypsin activation; B, trypsin activated medium of 3.7; C, 50:50 mixture of trypsin activated medium of 3.7 plus medium of RE2; D, 50:50 mixture of trypsin activated medium of 3.7 plus trypsin activated medium of RE2; E, conditioned medium of RE2 without trypsin activation; F, trypsin activated medium of RE2; G, 50:50 mixture of medium of 3.7 plus trypsin activated medium of RE2; H, medium of 3.7 plus medium of RE2. The amount of 3.7 conditioned medium was identical in all samples (4-H).

The mechanism by which members of the activated ras oncogene family can induce the complex metastatic phenotype after transfection into recipient cells (1, 2, 4–6) remains obscure. The transfected cell must be of the appropriate cellular background. For example, ras will induce NIH/3T3 cells to become tumorigenic and metastatic, but the same oncogene transfected into murine C-127 cells will confer tumorigenicity without metastases (4). The effect of ras on spontaneous metastases versus lung colonization by the i.v. route may also relate to the cellular background (17). Thus, the recipient cell must contribute the correct genetic elements which cooperate with the effects of the ras oncogene to result in the metastatic phenotype. However, it is clear that an abnormal karyotype is not a prerequisite for the metastatic phenotype to be induced. A variety of diploid cells can be induced to become metastatic by activated ras oncogene transfection (3, 4). Some metastatic transfectants remain diploid with no gross karyotypic alterations (18). It is possible that the transfected oncogene itself, or the oncogenic M, 21,000 protein induces a genetic instability leading to metastatic variants which are selected in vivo. If this is the case, it must occur very rapidly since metastases following s.c. injection were observed as early as passage three or four following ras transfection into rat embryo fibroblasts (6), and all of the metastatic ras-transfected REF continuously retained their metastatic potential for at least 40 passages. Colony forming efficiency in agar and degree of anchorage independent growth is not necessarily correlated with metastatic propensity in oncogene transformed or carcinogen transformed cells (6).

In Fig. 2, a variety of carcinogen transformed fibroblasts were shown to be fully tumorigenic (forming 1-cm tumors within 30 days), were not metastatic in nude mice. The protooncogene (4) can confer anchorage independence and in vivo tumorigenicity, but the transformed cells remain nonmetastatic (4). ras plus E1a REF transfectants are tumorigenic but nonmetastatic (6). In contrast, ras plus v-myc REF transfectants are fully metastatic. These data indicate that metastatic propensity clearly requires specific cellular alterations over and above those sufficient for tumorigenicity alone.

Even though the mechanism of ras induction of the metastatic phenotype is unknown, this system provides a new opportunity to study specific gene products which correlate with the induced phenotype. Undoubtedly, multiple gene products are required for progression to metastases. Any putative gene product necessary, but not sufficient, for completion of this progression (or biochemically linked to necessary gene products) must be expressed by all of the metastatic clones. One of many putative gene products associated with metastases is a protease which enables the tumor cell to traverse the continuous basement membranes, which separate tissue compartments and surround blood vessels, muscles, and nerves. As shown in Figs. 1 and 3, all of the metastatic transfectants secreted high levels of type IV (basement membrane) collagenolytic activity. Furthermore,
without exception, all the tumorigenic but nonmetastatic transfectants and carcinogen-transformed cells (Fig. 2) failed to produce significant amounts of this protease. The exactness of this correlation in these cells may be highly dependent on the fact that they differ in metastatic propensity for reasons unrelated to susceptibility to natural killer or macrophage cell killing in the nude mouse system (1). Certainly in syngeneic mice a tumor cell with high collagenolytic activity could be also highly immunogeneic and consequently not able to resist host defenses and initiate a metastatic focus. As shown in Table 2, the metastatic propensity did not correlate with PA production by these lines. Regulation of PA and collagenase may both be important for metastases. However, PA alone in the absence of collagenase appears insufficient for metastases.

Type IV collagen, the structural element of basement membranes, is resistant to classic collagenase, which degrades collagen I, II, and III. A collagenase that cleaves procollagen IV at a specific site has been identified in cell cultures derived from malignant tumors (7, 12, 14). This type IV-specific collagenase is a neutral metal proteinase of molecular weight of about 60,000-70,000. The proteinase is inhibited by metal chelators and serum, but not by N-ethylmaleimide, phenylmethanesulfonyl fluoride, or aprotinin. It is secreted in a latent form which can be activated by trypsin or plasmin.

Procollagen IV monomers are triple helical molecules that contain a noncollagenous globular knob at one end and the “7S” bow tie-shaped domain at the other end. Four type IV monomers can associate by cross-links and disulfide bonds to form a tetramer. The individual molecules are joined at the 7S domain, and from this junction the knob-like termini extend, as shown by electron microscopy (19). The 7S domain is at the NH₂ termini, and the tumor cell type IV-specific collagenase cleaves at a site 25% of the distance from this end. The tumor enzyme therefore has the ability to break apart the tetramer and potentially dissociate the type IV network in the basement membrane lamina densa.

The ras-transformed rat embryo fibroblasts secrete into the culture media a protease active against type IV collagen (Figs. 4 and 5) that has properties similar or identical to the previously described murine tumor cell (12, 14), and human mononuclear phagocyte (15) type IV-specific collagenase. The pH optimum, the cleavage products, and the inhibitor profile are identical. The REF collagenase is secreted in a latent form, similar to that found for mouse tumor cells and not in the active form as found for the normal human mononuclear phagocytes (14). The absence of secretion of type IV collagenolytic activity by diploid REF cells cotransfected with ras plus E1a is in parallel with the possible inhibitory effect of E1a on the metastatic phenotype (6). The absence of significant levels of this protease in the ras plus E1a cell-conditioned media is not due to the elaboration of possible protease inhibitors or to the absence of required specific activators (Fig. 6). The effect of the adenovirus type 2 E1a gene cotransfection appears to interfere with the cascade of genetic events promoted by the insertion of ras oncogene, and is not a rare event, since all the analyzed transfected clones exhibit the nonmetastatic low collagenase phenotype. As previously reported, cotransfection of ras plus E1a has no effect on the level of specific RNA transcripts for both oncogenes. In contrast to the results with E1a, all of the ras plus v-myc-transfected REF produced spontaneous and i.v.-induced metastases and secreted high levels of type IV collagenolytic activity (Fig. 3). It has been previously reported that the adenovirus type 2 E1a gene can interact with the transforming function of ras oncogenes and can modify cell surface antigens and increase susceptibility of cells to lysis by natural killer cells and macrophages, at least in the hamster system (20, 21). Whether or not immune cell lysis susceptibility is altered for the ras plus E1a-transfected REF cells, remains to be determined. Nevertheless, the alteration in collagenase expression demonstrates a gene product dramatically altered by the presence of E1a. The fact that the E1a reduction of metastases is also associated with the loss of collagenase IV implicates this protease as being biochemically linked to the metastatic cascade in this rodent model.

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