Vol. 137, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1986 Pages 161-166

COLLAGEN PROCESSING IN RAS-TRANSFECTED MOUSE MAMMARY EPITHELIAL CELLS

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Received April 10, 1986

A mouse mammary epithelial cell line (NMuMG), after transfection with the c-ras^{H} oncogene, forms invasive tumors in nude mice. NMuMG and NMuMG/p-ras^{H} cells produce similar amounts of collagen (mostly type IV) when grown on plastic. NMuMG cells respond to growth on collagen gels by increasing the rate of collagen synthesis and deposition by 100%, unlike NMuMG/p-ras^{H} cells which synthesize similar amounts of collagen whether grown on plastic or collagen gels. These results suggest that ras transformation partially inhibits the interaction between epithelial cells and the surrounding stroma that is necessary for basement membrane deposition in vivo and consequently may facilitate the invasion of the stroma by transfected cells.

Basement membranes are thin layers of extracellular matrix that separate epithelial structures from the surrounding stroma. One function of the basement membrane is to act as a barrier to the passage of both epithelial and mesenchymal cells (1). A characteristic of malignant breast tumors is loss of basement membrane in areas of invasion (2-4). Loss of basement membrane appears to be a necessary pre-requisite before malignant breast cells can invade the surrounding stroma and disseminate. The mechanism of the loss of basement membrane in malignant tumors is not entirely clear. In some cases, tumor cells may produce collagenolytic and proteolytic enzymes that are capable of degrading basement membranes (5). It has also been suggested that a block in the expression of the differentiated phenotype may result in loss of the ability to synthesize or deposit a basement membrane (6). Transformation of cells with oncogenes is known to induce tumorgenicity and, in some cases, malignancy (7-9). However, the role played by oncogenes at each stage of the metastatic process is largely

0006-291X/86 $1.50

unknown. Here, we investigate the effects of transfecting mammary epithelial cells with the EJ-ras^H oncogene on the ability to deposit type IV collagen, a major structural component of basement membranes.

EXPERIMENTAL PROCEDURES

Cell Culture. The transfection of NMuMG cells with pSV2neo and a plasmid containing the activated c-ras^H oncogene (p-ras^H) which had been molecularly cloned from the EJ human bladder carcinoma cell line (10) has been described previously (11). For control studies, NMuMG cells were transfected with pSV2neo and a plasmid containing human placental DNA (11). Both cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 5% dialyzed fetal calf serum, 50ng/ml insulin, 50ng/ml hydrocortisone, and 50μg/ml ascorbic acid. Cells were grown on collagen gels (rat tail collagen) as described previously (12).

Immunofluorescence Studies. Immunofluorescence staining of cell lines with an antibody to type IV collagen was carried out as described previously (12).

Collagen Synthesis and Degradation. Confluent cultures of cells were labeled with [^{14}C]proline (2μCi/ml) for 24 h. After addition of protease inhibitors, proteins were precipitated from the medium by adding an equal volume of 10% trichloracetic acid (TCA)-0.5% tannic acid. The cell layers were washed 3 times with phosphate-buffered saline and extracted with 5% TCA-0.25% tannic acid. TCA-soluble fractions were combined, extracted twice with ether and lyophilized. The TCA-insoluble fractions were washed 3 times with water and lyophilized. The TCA-soluble and insoluble fractions were hydrolyzed with 6M HCl at 110^{o}C for 24 h. Prior to hydrolysis, [^{3}H]hydroxyproline (10,000 cpm) was added to each fraction to monitor recovery of hydroxyproline. The hydrolyzates were lyophilized and chromatographed on a Biotronic LC2000 amino acid analyser equipped with a stream splitter, and the radioactivity in 3- and 4-hydroxyproline and proline determined. The [^{14}C]proline used in these experiments was purified on Dowex-2, and contaminants chromatographing in the hydroxyproline region accounted for less than 0.01% of the total radioactivity. A sample of [^{14}C] proline was hydrolyzed with each batch of samples to determine background levels of radioactive material eluting in the hydroxyproline position.

RESULTS

Immunofluorescence studies. Immunofluorescence staining of cultures of confluent cells grown on plastic with an antibody to type IV collagen revealed the presence of an extracellular matrix containing type IV collagen in both ras-transfected and control cultures (cells transfected with placental DNA). The intensity of staining was similar for both cell lines (Fig.1). Neither cell line stained with an antibody to procollagen I (data not shown).

Collagen synthesis and degradation. The amount of collagen produced by NMuMG/p-ras^H and control NMuMG cells is similar when the cells are grown on plastic as measured by the incorporation of hydroxyproline into TCA-insoluble material (Table 1). NMuMG/p-ras^H cells accumulate slightly more collagen in the cell layer and less in the culture medium that control cells. Using the formula

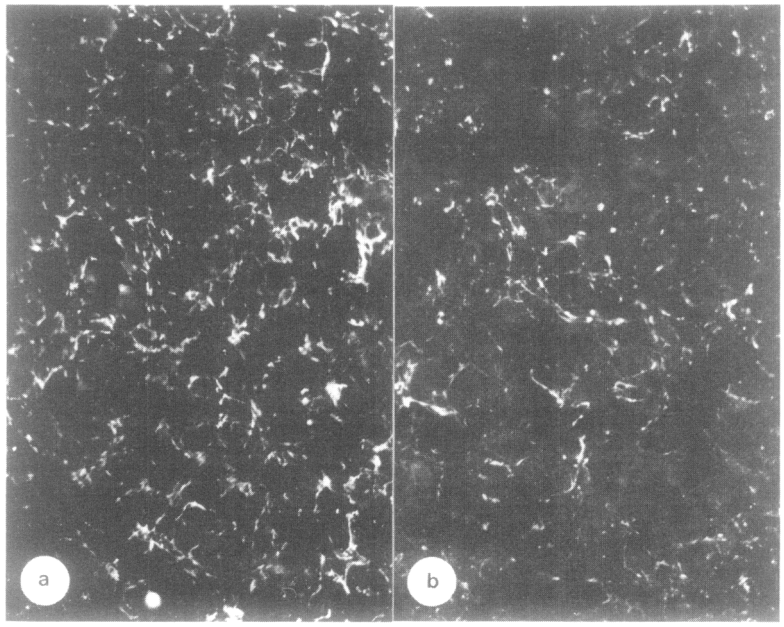

Fig.1. Immunofluorescence localization of type IV collagen in cultures of (a) NMuMG cells and (b) NMuMG/p-ras^{H} cells growing on plastic. Note the extracellular fibres staining with the antibody in both cell lines, x 550.

described by Holderbaum and Ehrhart (13), collagen accounts for 0.56% of the total protein synthesized by NMuMG/p-ras^{H} cells and 0.62% of the total protein synthesized by control cells. However, the total amount of hydroxyproline synthesized by NMuMG/p-ras^{H} cells is 40% higher than NMuMG cells. The increased production of hydroxyproline is largely accounted for by an increase in TCA-soluble

Table 1 Collagen synthesis and degradation in NMuMG and NMuMG/p-ras^{H} cells growing on plastic

	hydroxyproline (cpm/10^6cells)	
	NMuMG (% of total)	NMuMG/p-ras^{H} (% of total)
Cells, TCA-insoluble	6,411 ± 248 (36.4%)	7,659 ± 451 (30.6%)
Medium, TCA-insoluble	3,921 ± 119 (22.3%)	2,544 ± 94 (10.1%)
Cells & Medium, TCA-soluble	7,275 ± 622 (41.3%)	14,868 ± 1,167 (59.3%)
Total hydroxyproline[1]	17,607 ± 547 (1)[3]	25,071 ± 1,426 (1.42)
Collagen hydroxyproline[2]	10,332 ± 215 (1)	10,203 ± 388 (1)
Total proline[4]	0.59×10^5 ± 0.04×10^5	0.65×10^5 ± 0.04×10^5

[1] TCA-soluble + TCA-insoluble hydroxyproline
[2] TCA-insoluble hydroxyproline
[3] Relative proportion
[4] Cellular + medium TCA-insoluble proline

Table 2 Collagen synthesis and degradation in NMuMg and NMuMG/p-ras^{H} cells growing on collagen gels

	Hydroxyproline (cpm/10^6 cells)	
	NMuMG (% of total)	NMuMG/p-ras^{H} (% of total)
Cells, TCA-insoluble	10,002 ± 634 (41.6%)	5,580 ± 411 (43.1%)
Medium, TCA-insoluble	2,541 ± 327 (10.6%)	783 ± 104 (6.1%)
Cells & Medium, TCA-soluble	11,484 ± 1,017 (47.8%)	6,579 ± 620 (50.8%)
Total hydroxyproline[1]	24,027 ± 1,135 (1)[3]	12,942 ± 607 (0.51)
Collagen hydroxyproline[2]	12,543 ± 720 (1)	6,363 ± 298 (0.51)
Total proline[4]	0.39×10^5 ± 0.02×10^5	0.38×10^5 ± 0.03×10^5

[1] TCA-soluble + TCA-insoluble hydroxyproline
[2] TCA-insoluble hydroxyproline
[3] Relative proportion
[4] Cellular + medium TCA-insoluble proline

hydroxyproline in NMuMG/p-ras^{H} cultures. TCA-soluble hydroxyproline is only produced by the degradation of collagen. Consequently, NMuMG/p-ras^{H} cells degrade 60% and control NMuMg cells degrade 40% of their newly synthesized collagen. This degradation probably occurs intracellularly as all labeling procedures were carried out in the presence of 5% serum effectively inhibiting the activation of any collagenolytic enzymes.

When the cells are grown on collagen gels, there is a marked difference in the amount of collagen produced, NMuMG/p-ras^{H} cells only producing 50% of the amount produced by control NMuMG cells (Table 2). Collagen now accounts for 0.59% of the total protein synthesized by NMuMG/p-ras^{H} cells and 1.15% of the total protein synthesized by control cells. The total hydroxyproline synthesized by NMuMG/p-ras^{H} cells is also reduced to 50% of control levels with both cell lines degrading 50% of newly synthesized collagen. The amount of [^{14}C]proline incorporated into TCA-insoluble protein is similar in the two cell lines indicating that any difference in the intracellular proline pools is unlikely to have influenced the results.

DISCUSSION

Growth of cells on collagen gels is known to result in increased deposition of basement membrane constituents (14, 15). NMuMG cells are similar, in this

respect, in that they accumulate 100% more collagen when grown on collagen gels. As the total amount of hydroxyproline synthesized also increases by approximately 100% (relative to total protein synthesis) and there is no decrease in collagen degradation, the increased deposition of collagen must result from increased synthesis. Most of the collagen synthesized by NMuMG cells is probably type IV collagen as these cells did not stain with an antibody to type 1 procollagen and 80% of the collagenous proteins secreted by this cell line did not bind to DEAE-cellulose at pH 8.6 (16).

Unlike NMuMG cells NMuMG/p-ras^{H} cells are unable to respond to growth on collagen gels by increasing their rate of collagen production. This results from a failure to increase the rate of collagen synthesis as both net collagen production and total hydroxyproline synthesis are similar in NMuMG/p-ras^{H} cells grown on plastic and collagen gels relative to total protein synthesis. Although we have not assayed these cell lines for the production of extracellular collagenolytic enzymes, our results indicate that the failure of NMuMG/p-ras^{H} cells to deposit increased amounts of collagen when grown on collagen gels is not due to increased degradation of collagen. Indeed, TCA-soluble hydroxyproline production decreases from 60% of the total hydroxyproline synthesized in cells growing on plastic to 50% in cells growing on collagen gels. The production of TCA-soluble hydroxyproline probably results from the intracellular degradation of collagen, a process that occurs in most cultured cells (17), and is comparable to the extent of collagen degradation previously reported in a rat mammary epithelial cell line (15).

After injection into nude mice, control NMuMg cells form benign cystadenomas with a tumor incidence of 20% and a latency period of 6.5 weeks. NMuMG/p-ras^{H} cells, however, form invasive carcinomas with a tumor incidence of 100% and a latency period of 2.5 weeks (11). It has also been reported that high passage NMuMG cells, unlike low passage cells, have a reduced ability to form a basal lamina when grown on collagen gels in vitro and form invasive tumors which contain fragmented basement membrane deposits when injected into nude mice (18). These results suggest that abnormal basement membrane deposition permits tumor cells to invade the surrounding stroma and that ras transfection contributes to these events

by partially inhibiting the interaction between epithelial cells and the surrounding stroma that is necessary for basement membrane deposition *in vivo*. Ras transformation of bladder epithelial cells has recently been shown to completely inhibit the extracellular deposition of type IV collagen and laminin (19), indicating that ras transformation of epithelial cells from different tissues may result in a spectrum of basement membrane changes.

ACKNOWLEDGEMENTS

Michael J Warburton was supported by a grant from the Cancer Research Campaign.

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