ABSTRACT

Conditioned medium derived from the colon cancer cell lines was ineffective in solubilizing immobilized radiolabeled laminin. However, substantial degradation was observed in the presence of plasminogen and could be largely blocked by preincubation with polyclonal anti-urokinase antibody. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the solubilized products generated either by the conditioned medium or by authentic urokinase supplemented with plasminogen yielded identical results. Analysis of the spent medium for urokinase by an enzyme-linked immunosorbent assay method revealed a similar profile to that achieved with the laminin degradation assays for the six cell lines tested. However, Northern analysis of urokinase-specific mRNA indicated that protein levels could not be entirely predicted by steady-state levels of the transcript.

In a previous study, undifferentiated colon cancer cell lines expressed larger amounts of the plasminogen activator into the conditioned medium compared with their well-differentiated counterparts. However, these earlier studies were performed using cells grown in defined medium which lacked epidermal growth factor (EGF). EGF has been reported to affect plasminogen activator levels. Consequently, to investigate the role of EGF in the modulation of urokinase protein/activity, cell types representative of well- and poorly differentiated colon cancer were examined for their sensitivity of expression to this growth factor. In the absence of EGF, primitive cell types secreted, on average, 5 times more urokinase than their well-differentiated counterparts. In response to EGF, however, well-differentiated cell lines exhibited 4- to 6-fold increases in these parameters while the primitive cell lines were refractory to the peptide. Consequently, the differences in urokinase protein expressed by the well- and poorly differentiated groups of cells were abolished by the presence of EGF.

The expression of a well-differentiated phenotype by colon cancer cell types In vivo probably depends to some extent on laminin within a basement membrane. The data presented herein are consistent with the idea that depletion of this glycoprotein from a basement membrane by urokinase-dependent mechanisms may contribute to the undifferentiated phenotype seen with many of these malignancies.

INTRODUCTION

The presence of an intact basement membrane appears to be a crucial factor for the differentiation of a number of cell types of diverse origin (1, 2). This laboratory recently reported that "ECM-like material" was also capable of modulating the behavior of colon carcinoma cells in culture (3). Undifferentiated (primitive) colon carcinoma cells which were incapable of producing extracellular matrix components were, however, responsive to colon-derived substrata material (3). By various criteria including morphological change and sensitivity to the antiproliferative peptide TGF-β, ECM-like material appeared to cause a partial shift in the characteristics of the primitive colon carcinoma cells towards that displayed by the well-differentiated cell types (3). This phenomenon is hereby referred to as "maturation." Characterization of the colon deposits revealed the presence of the ECM component laminin. Moreover, similar changes in the behavior of the undifferentiated colon cells could be brought about by subculturating them onto laminin (4, 5).

These findings have led us to the view that an impaired extracellular matrix structure depleted of laminin may be one of a series of events which culminates in a primitive colon cancer cell. There are reports which indicate that less laminin is present in basement membrane structures associated with colonic adenocarcinomas compared with normal mucosa or benign lesions (6, 7). A compromised basement membrane may be a consequence of increased turnover of such components as laminin and fibronectin or, alternatively, may be a result of decreased synthesis. The former possibility was considered in this study since there is abundant evidence documenting the elevated expression of various proteases such as collagenase, elastase, stromelysin, urokinase, and cathepsin B in separate tumor tissues (8, 9). This study attempts to define which, if any protease(s) elaborated by cultured colon carcinoma is involved in the destruction of the basement membrane glycoprotein, laminin. In this respect, we previously reported the presence of urokinase in the conditioned medium derived from a number of cultured colon carcinoma cell lines (10). However, no evidence was presented to indicate whether the plasminogen activator was enzymatically active. More important, these studies did not address the function of this plasminogen activator in colonic malignancies. Urokinase may participate in a number of biological events including ECM dissolution (11), growth factor activation (12), stimulation of proliferation (13), and neutrophil mobilization (14). Also, the expression of urokinase by colon cancer, in vitro, does not necessarily exclude possible roles for other laminin-degrading enzymes, such as cathepsin B and stromelysin (8).

The salient features of this study are 2-fold. (a) The degradation of exogenous radioactive laminin by colon carcinoma cell lines is primarily a function of urokinase expression by these cells. (b) Elevated urokinase secretion by the undifferentiated colon carcinoma cells is independent of exogenous EGF. In contrast, high expression of this plasminogen activator by well-differentiated colon cell lines is only seen in the presence of this growth factor. These data argue for an increased laminin-degrading capacity of the primitive group of cells which is due in part to their growth factor autonomy. Insofar as laminin may be required for maturation of colon carcinoma, in vitro, its removal from a basement membrane, in vivo, by urokinase-dependent mechanisms could be one of many events which culminate in an undifferentiated cell type.

MATERIALS AND METHODS

Plasminogen and insulin were supplied by Sigma Chemicals, St. Louis, MO. Laminin and EGF were purchased from Collaborative Research, Bedford, MA. The plasmin-containing urokinase cDNA was obtained from American Type Culture Collection, Rockville, MD.
High-molecular-weight urokinase ($M_0, 55,000$) was a generous gift from Dr. Genesio Murano, Department of Drugs and Biologics, Bethesda, MD. Dr. Joffre Baker, Lawrence, KS, kindly supplied the polyclonal anti-urokinase antibody. ELISA antibodies were obtained from Dr. Désiré Collen, Leuven, Belgium.

**Tissue Culture.** The human colon carcinoma cell lines CBS, GEO, FET, HCT 116, HCT 116b, and RKO have been extensively characterized elsewhere (15, 16). All cell lines were carried in McCoy's Medium 5A supplemented with 5% fetal bovine serum (Gibco, Grand Island, NY) as described previously (10). The cells were subcultured at 80% confluency with 0.125% trypsin for the well-differentiated CBS, GEO, and FET or 0.05% trypsin for the undifferentiated (primitive) HCT 116, HCT 116b, and RKO in the presence of 3 mM EDTA. Medium changes were performed every fourth day.

Collection of conditioned medium was as follows. Cells were passed into Falcon 35-mm (s) dishes, and at approximately 80% confluency, the medium was replaced with McCoy's Medium 5A containing 20 µg/ml of insulin, 10 ng/ml of EGF, and 4 µg/ml of transferrin (serum-free medium). The cultures were maintained for an additional 3 days, the spent medium was harvested and stored at $-20°C$, and cells were enumerated.

For mRNA analysis, colon carcinoma cells were propagated in serum-containing medium in duplicate 75-cm² CoStar flasks.

**Laminin Degradation Assays.** Laminin (150 µg) was iodinated at room temperature by a standard chloramine T method (17). Radiolabeled glycoprotein was dialyzed extensively at 4°C against PBS and finally equilibrated with 50 mM carbonate buffer (pH 9.6). The radioactive active laminin was dispensed into radioimmunoassay tubes (Sarstedt, Princeton, NJ) and shaken for 12 h. The tubes were washed 2 times with PBS containing 1 mg/ml of bovine serum albumin and blocked with the same for 1 h at room temperature. Routinely, individual tubes bound approximately $5 \times 10^6$ dpm of radioactive laminin.

Conditioned medium was diluted 1:50 in 25 mM Tris-HCl buffer (pH 7.5) and added to $^{125}$I-labeled laminin-coated tubes. One-half of the tubes was supplemented with 10 µg/ml of plasminogen with the remaining tubes being incubated in the absence of this zymogen. Spontaneous release of $^{125}$I-labeled laminin was monitored with a 1:50 dilution of fresh serum-free medium. Incubation of the radioactive laminin-coated tubes with either urokinase or plasminogen alone did not result in the solubilization of the glycoprotein. All incubations were carried out at $37°C$ for 3 h with gentle stirring. Solubilized radioactive laminin was quantified by counting aliquots of the supernatant for radioactivity or, alternatively, was analyzed on a 12.5% SDS-PAGE gel under reducing conditions (18).

**Determination of Urokinase Levels in the Spent Medium.** Conditioned medium was analyzed for urokinase using an ELISA method as described previously (3, 19). The ELISA detects high- ($M_0, 55,000$) but not low- ($M_0, 33,000$) molecular-weight urokinase and has a sensitivity of 0.1 ng/ml.

**Northern Analysis of Steady-State, Urokinase mRNA Levels in Colon Carcinoma Cell Lines.** Total cellular RNA was extracted from 80% confluent colon carcinoma cell lines using guanidinium isothiocyanate and centrifuged at 150,000 × $g$ on a 5.7 M cesium chloride cushion (20). The RNA was electrophoresed in a formaldehyde-agarose gel and transferred to nylon (Schleicher and Schuell, Keene, NH) by capillary action. The filter was probed using a multiprimed radiolabeled (Amersham, Arlington, Heights, IL) 1.5-kilobase cDNA probe specific for the urokinase transcript (22). Filters were washed for equivalent loading by reprobing with a cDNA fragment specific for the GAPDH message.

**RESULTS**

**Solubilization of Radioactive Laminin by Colon-derived Conditioned Medium.** In the absence of thezymogen, plasminogen, spent medium from the 6 colon cell lines examined was unable to solubilize the immobilized radioactive substrate (Fig. 1A) even after prolonged exposure (15 h). However, supplementing the conditioned medium with plasminogen (Fig. 1A) caused a substantial release of radiolabeled material into the supernatant indicating the importance of one of the plasminogen activators in this process. The GEO and the RKO cell lines were relatively poor stimulants in this respect (Fig. 1A), while conditioned medium from CBS, FET, HCT 116, and HCT 116b caused extensive solubilization in a 3-h period. The ability of the spent medium to release radioactive material from the tube was effectively blocked (80% of the solubilizing activity) by preincubation of the medium with polyclonal anti-urokinase antibody (Fig. 1B) but not by an irrelevant antibody.

**SDS-PAGE analysis of the solubilized material followed by autoradiography (Fig. 2) revealed identical profiles for the colon carcinoma cell lines examined. More important, the autoradiogram of the positive control in which authentic urokinase and the zymogen, plasminogen, were coincubated with $^{125}$I-labeled laminin was essentially the same as that obtained when spent medium was supplemented with plasminogen.

**Determination of Urokinase Levels by an ELISA.** Analysis of
UROKINASE-MEDIATED LAMININ DEGRADATION IN COLON CANCER

Fig. 2. Analysis of labeled laminin degradation products. Conditioned medium diluted 50-fold was incubated with plasminogen in radioactive laminin-coated tubes at 37°C for 4.5 h. Aliquots of the supernatants were denatured in the presence of 5% β-mercaptoethanol. Equivalent amounts of radioactivity (5 × 10^6 dpm) were electrophoresed in a 12.5% polyacrylamide gel. The gel was subjected to autoradiography. Lanes 1 and 2, 10^6 and 5 × 10^6 dpm of intact radioactive laminin, respectively; Lanes 3 and 4, 5.0 ng/ml of authentic urokinase supplemented with plasminogen: Lanes 5 and 6, CBS; Lanes 7 and 8, FET; Lanes 9 and 10, HCT 116; Lanes 11 and 12, HCT 116b. Molecular weight markers are on the right. The experiment was performed twice.

Fig. 3. Determination of urokinase levels in the conditioned medium. Spent medium was diluted 1/25, and the level of urokinase was determined from a standard curve constructed with a range (0.1 to 5.0 ng/ml) of urokinase concentrations. Columns, average values of 4 separate determinations; bars, SD.

the conditioned medium for this plasminogen activator (Fig. 3) revealed a similar pattern to that obtained with the laminin degradation assays (Fig. 1A). Both GEO and RKO were low secretors of urokinase, while CBS, FET, HCT 116, and HCT 116b were more active in this respect.

Northern Analysis of Urokinase Steady-State mRNA Levels. Elevated levels of secreted urokinase by CBS, FET, HCT 116, and HCT 116b were associated with a strong signal generated by Northern blotting of the respective RNAs (Fig. 4). The other hand, GEO, which was one of the low secretors of urokinase, expressed a level of transcript which was below the detection limits of this hybridization assay. However, a complete correlation of urokinase message with protein levels in the conditioned medium could not be achieved with all of the surveyed colon carcinoma cell lines (Fig. 4). The RKO cell line, which was also a low secretor of this plasminogen activator, was found to express a relatively high amount of the urokinase message.

EGF Induction of Urokinase Protein in Well- but not Poorly Differentiated Cells. We previously reported that urokinase secretion in cultured colon cancer cells was inversely linked with differentiation status (3, 10). In the earlier studies, EGF was absent from the serum-free medium. It has now been established that this growth factor is a requirement for the continued maintenance of the well-differentiated colon cancer cell lines under serum-free conditions. In contrast, in the absence of serum, the primitive cell lines will propagate indefinitely without EGF probably due to adequate autocrine stimulation by endogenous TGF-α (24). Table 1 illustrates the response in urokinase level/activity to EGF in typical well-differentiated cell lines CBS and GEO and in RKO and HCT 116, cell lines representative of the primitive group. In the absence of EGF, conditioned medium from the primitive cells contains, on average, 5 times as much urokinase than spent medium collected from the well-differentiated cells. A similar trend is observed with data generated by the laminin degradation assay. When both groups of cells are cultured in the presence of EGF, however, these differences are abolished. This is a consequence of an increased urokinase output in response to EGF by the well-differentiated cells with no change occurring in this parameter for the primitive cell lines.
DISCUSSION

We previously reported that primitive colon cancer cells which were incapable of producing their own laminin (3) were, however, responsive to this extracellular matrix glycoprotein. The changes effected by laminin were consistent with the idea that the glycoprotein was necessary for “driving” the primitive colon cancer cells towards a well-differentiated cell type. It was not possible, however, to achieve a well-differentiated colon cancer cell line by cultivation of primitive cells with laminin alone. Clearly, other biological mechanisms are imperative for a differentiation program to be completed.

Irrespective of this, it can be argued that either a decreased capacity to deposit laminin in a basement membrane structure or an increased ability to remove it, via proteolytic action, may contribute to an impaired structure which is partly responsible for defective maturation. The study, herein, demonstrates that urokinase elaborated by cultured colon cancer cells may, via its activation of exogenous plasminogen, cleave radiolabeled laminin into multiple fragments. In human subjects, the concentration of circulating plasminogen is approximately 200 μg/ml (25). Thus it is unlikely that urokinase-mediated plasmin production by colon cancer in vivo would be limited by the abundance of the zymogen. An unchecked level of plasmin could consequently degrade laminin within an underlying basement membrane. This proposal is given credence by immunofluorescence studies (6, 7) of basement membranes derived from normal colonic mucosa and adenocarcinoma. The amount of laminin associated with this structure derived from malignant tissues was markedly reduced in comparison with healthy tissues.

Although cathepsin B or stromelysin activities were not measured directly, it is unlikely that these enzymes play a major role in the degradation of exogenous radiolabeled laminin. Thus, turnover of radioactive laminin by the 6 colon carcinoma cell lines was entirely dependent on the presence of plasminogen. Further, polyclonal, anti-urokinase antibody largely blocked the degradation of the 125I-labeled glycoprotein. Perhaps the most compelling evidence implicating urokinase in this process, however, was that the SDS-PAGE profiles of 125I-labeled laminin breakdown, generated either by authentic urokinase or conditioned medium supplemented with plasminogen, were identical.

Although other laboratories including ours have reported elevated activity in the malignant colon (10, 26, 27), the role of this plasminogen activator in this cancer has not been addressed. Urokinase may participate in a number of cellular events including ECM dissolution (4), activation of the growth factor TGF-β (12), stimulation of proliferation (13), and neutrophil chemotaxis (14). The data presented in this study indicate that one of the major actions of urokinase in colon cancer in vitro is the turnover of exogenous laminin which in vivo is an integral constituent of the basement membrane. However, it is important to note these studies do not address the function of the urokinase receptor (10, 28) in the laminin-degrading actions of this plasminogen activator. Considering the cellular receptor in such a process would require plating of the colon cancer cells directly onto an ECM. We have shown that ECM-like material may affect both urokinase secretion (3) and the urokinase binding site. Therefore, as an alternative, conditioned medium from the cell lines was utilized to determine their proteolytic capacity.

The lack of an inverse relationship between urokinase levels/activity and differentiation differs from our previous results (10). In the earlier study, serum-free medium utilized for the collection phase lacked EGF, while in the present investigation, this growth factor was included. These conditions were altered in light of studies by this laboratory (23) demonstrating continued proliferation of the well-differentiated CBS, GEO, and FET cells in serum-free medium supplemented with EGF. The undifferentiated HCT116, HCT116b, and RKO, however, did not require EGF (29), this possibly being a result of autocrine production of TGF-α by these colon cells abrogating their responsiveness to the exogenous growth factor (24). TGF-α is considered to exert the majority, if not all, of its biological effects via interactions with the EGF receptor (30). These findings may throw some light onto the apparent discrepancy between the urokinase data generated in this study and in an earlier report (10). The primitive cell lines in the absence of EGF secrete, on average, 5 times the amount of urokinase compared with the well-differentiated cell types (Table 1) (10). However, inclusion of this growth factor into the culture medium elevates secretion rates by the well-differentiated CBS and GEO 4- to 6-fold with no change in the capacity of the primitive cell lines. The net result of this experimental manipulation is the lack of correlation of urokinase secretion with the primitive state. Thus, it would appear that the potential of urokinase secretion as a marker of transformation is highly dependent on both the growth factor environment and the autonomous nature of the cell type under investigation. Clearly, future studies utilizing this parameter as an index of the primitive state should take these factors into consideration.

It is tempting to speculate that the difference in urokinase levels observed between well- and poorly differentiated tumors in various malignancies (31) is linked to growth control. Thus, in the well-differentiated cancers, a reduced output of the plasminogen activator could reflect an inconsistent stimulation of these cells by circulating EGF. On the other hand, persistent stimulation of the primitive cancer cells by their own autocrine growth factors (32, 33) (e.g., TGF-α) could constitute a strong internal “drive mechanism” for urokinase expression. If urokinase production by colon cancer cells is partly a result of autocrine growth factor stimulation, restoration of normal growth control (i.e., the acquisition of sensitivity to exogenous growth factors) may have the additional benefit of rendering urokinase production by these cells subservient to external signals. This could ultimately translate into a reduced output of the plasminogen activator.

It is unlikely that elevated urokinase expression by the undifferentiated colon cancer cells is solely responsible for the removal of laminin from a basement membrane. We have noted a 10-fold higher expression of urokinase receptor number (10) in these cells compared with their well-differentiated counterparts. It is very possible that this increased display of binding sites by the primitive cells contributes to laminin dissolution (28). The receptors could serve to localize the plasminogen activator in the immediate vicinity of the cells (28).

The level of urokinase steady-state mRNA cannot entirely account for the protein levels in the conditioned medium. Thus, a relatively large amount of urokinase mRNA transcript derived from RKO cells is associated with low plasminogen activator levels as assessed in the laminin degradation assays and with the ELISA. One possibility is that the urokinase secreted by these cells is rapidly inactivated. Alternatively, secretion of the plasminogen activator by these cells may occur in a manner independent of intracellular levels. This latter concept is akin to that proposed by Blum et al. (34). Protein secretion of the differentiation marker, lactalbumin, by rat mammary cells represented a highly regulated phenomenon.
In summary, we have demonstrated that the plasminogen-dependent degradation of exogenous laminin by conditioned medium derived from 6 colon cancer cell lines largely reflects the expression of urokinase in the conditioned medium. In this regard, the undifferentiated colon cancer cell types may have the advantage, since the elevated secretion of urokinase by these cells is independent of exogenous growth factors. In contrast, to boost the amounts of urokinase in the conditioned medium, the well-differentiated cell lines secrete low levels of the plasminogen activator and must be stimulated by exogenous EGF to boost the amounts of urokinase in the conditioned medium. Laminin has been shown to induce maturation of primitive colon cancer cells and therefore may be one of many factors required for the culmination of a well-differentiated colon cancer cell type. Consequently, the removal of laminin from a basement membrane structure in vivo via urokinase-dependent mechanisms could contribute to the undifferentiated state observed with many of these malignancies. On the basis of these findings, it will be of interest in future studies to determine whether anti-urokinase agents have any effect on the maturation of cultured colon carcinoma.

ACKNOWLEDGMENTS

We are indebted to Drs. Désiré Collen, Leuven, Belgium, and Joffre Baker, Lawrence, KS, for the generous gift of monoclonal and polyclonal antibodies. The authors wish to thank Germaine Florent for her excellent technical assistance.

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Examination of Urokinase Protein/Transcript Levels and Their Relationship with Laminin Degradation in Cultured Colon Carcinoma


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