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Amphiregulin and Hepatocyte-Derived Extracellular Matrix Regulate Proliferation and Autocrine Growth Factor Expression in Colon Cancer Cell Lines of Varying Liver-Colonizing Capability

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Abstract We studied the effect of two members of the epidermal growth factor (EGF) family—amphiregulin and heparin-binding EGF-like growth factor (HB-EGF)—on cell proliferation, growth factor and growth factor receptor expression, and cell differentiation in two human colon cell lines of varying liver-colonizing potential. The effect of amphiregulin and HB-EGF was assessed both in cells grown on plastic, as well as on cells grown on hepatocyte-derived extracellular matrix (ECM). We found that both colon cell lines were sensitive to HB-EGF stimulation of cell proliferation. Amphiregulin inhibited cell proliferation in KM12 cells and stimulated the strongly metastatic cell line KM12SM to a slight extent. When the cells were cultured on hepatocyte-derived ECM, amphiregulin inhibited the weakly metastatic KM12 and stimulated the growth of KM12SM. HB-EGF synergistically acted with hepatocyte-derived ECM to enhance cell proliferation in both colon cell lines. Expression of ligands of the EGF family, such as transforming growth factor-α (TGF-α) and amphiregulin, was decreased in both cell lines when cultured on ECM. Hepatocyte-derived ECM decreased expression of cripto in KM12 and increased it in KM12SM cells. Neither cripto nor TGF-α mRNA levels was affected by growing the cells in the presence of amphiregulin. However, amphiregulin increased expression of its own mRNA in the weakly metastatic KM12 and decreased it in the strongly metastatic KM12SM when the cells were cultured on plastic. Amphiregulin and HB-EGF stimulated expression of erb-B2 in both cell lines cultured on plastic. Surprisingly, when the cells were grown on hepatocyte-derived ECM, amphiregulin inhibited erb-B2 expression in both cell lines. We observed no effect of amphiregulin on cell differentiation as assessed by alkaline phosphatase expression. Our studies demonstrate one mechanism that could play a role in site-specific metastasis. We found an inhibitory response to an autocrine growth factor in the context of hepatocyte-derived ECM in a weakly metastatic cell and a stimulatory effect of the same growth factor when strongly metastatic cells were cultured on the same ECM. J. Cell. Biochem. 76:332–340, 1999.

Key words: amphiregulin; colon cancer; extracellular matrix; hepatocytes

The phenomenon of site-specific metastasis was described in 1889 by Paget, who suggested the "seed and soil" hypothesis and proposed that the newly colonized organ must be a fertile soil for tumor cells. The local milieu of the colonized organ can dictate the establishment and growth of tumor cells. These metastatic cells are able specifically to recognize and attach to local extracellular matrix (ECM) components and cells of the organ [Nicolson, 1989; Zvibel and Kraft, 1993]. Similarly, each organ secretes paracrine growth factors that can either stimulate or inhibit the growth of the tumor cells [Nicolson and Dulski, 1986; Radinsky, 1995a].

In recent studies, we showed that liver ECM stimulated cell proliferation in colon cancer cell lines of varying liver-colonizing ability. This effect was mediated by increased expression of members of the epidermal growth factor (EGF) receptor family [Zvibel et al., 1998a].

Colon carcinoma cells have been shown to secrete several autocrine growth factors [Cia-

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rdello et al., 1991]. In particular, members of the EGF family, both receptors and ligands, were found to play a crucial role in colon carcinomas. The number of EGF receptors were found to correlate with colon cancer metastasis to the liver [Radinsky, 1995b]. Analysis of human colon cancers and cell lines showed that most of them express the EGF receptor ligands TGF-α, amphiregulin (AR) and cripto [Ciardello et al., 1991]. AR has been found to be present in a large number of both malignant and benign colon tumors, particularly in well-differentiated tumors, but was present as well in the normal colon tissue [Saeki et al., 1992]. AR was either inhibitory or stimulatory or had no effect on cell proliferation in various carcinoma cell lines [Shoyab et al., 1988]. AR was found to act as an autocrine growth factor in colon carcinoma cell lines. Blocking its action by antibodies reduced cell proliferation in vitro [Johnson et al., 1992].

We found AR to be expressed by human colon cell lines of varying liver-colonizing ability. Our goal was to investigate the role of AR and HB-EGF, another member of the EGF family, by themselves or within the context of hepatocyte-derived ECM, in cell proliferation, differentiation, and autocrine growth factor expression in two colon cell lines with varying liver-colonizing ability.

MATERIALS AND METHODS

Cell Lines

The cells used in our studies were the low metastatic cell line KM12 (provided by Dr. Michael Sela, Weizmann Institute, Rehovot, Israel) and its liver colonizing derivate, KM12SM (provided by Dr. J. Fidler, M.D. Anderson Cancer Center, Houston, TX).

Cell Culture

Cell lines were grown in serum-supplemented medium (SSM) composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS)(Biological Industries, Beit Haemek, Israel), 100μg/ml penicillin, and 100 μg/ml streptomycin. AR, EGF, and heparin-binding EGF-like growth factor (HB-EGF) were added at a dose of 10 ng/ml. For the experimental studies, the cell lines were plated in SSM, then changed after 4 h to a medium described by Block et al. [1996] with slight modifications and termed hormonally defined medium (HDM). This HDM consisted of DMEM supplemented with 100 μg/ml penicillin/streptomycin, 2 mg/ml bovine serum albumin (BSA), 610 μg/ml nicotinamide, 740 ng/ml $ZnSO_4$:$7H_2O$, 20 ng/ml $CuSO_4$:$5H_2O$, 5 mM glutamine, 5 μg/ml insulin, 5 μg/ml iron-saturated transferrin, 5 ng/ml selenous acid, and 10^{-7} M dexamethasone. All the medium additives were obtained from Sigma Chemical Co. (St. Louis, MO).

Primary Cultures of Rat Hepatocytes

Primary cultures of rat hepatocytes were established by perfusing the liver of adult Wistar rats, using the method of Berry and Friend [1969]. The cells were plated on collagen I-coated plates and in HDM. Hepatocytes were plated at a density of 6×10^6 cells per 10-mm^2 dishes, 2×10^6 cells per well in 6-well plates and 2×10^5 cells per well in 24-well plates. After 5 days, hepatocyte plates were treated for 10 min at room temperature with 0.5% Triton X-100, then washed with phosphate-buffered saline (PBS). This treatment removes the cells and leaves the ECM they deposited on the plates.

Primary Cultures of Fetal Rat Fibroblasts

Rat embryos from pregnant Fisher rats gestation day 13 were cut into small pieces with a scalpel and the cells digested with 0.5% trypsin solution in PBS. After 20–30 min of digestion at 37°C, the cells were passed through a mesh and washed twice with DMEM supplemented with 10% FCS. A total of 300,000 cells were plated in 24-well plates coated with collagen type I. After 5 days, hepatocyte plates were treated for 10 min at room temperature with 0.5% Triton X-100 and then washed with PBS. This treatment removes the cells and leaves the ECM they deposited on the plates.

Growth Curves

Colon cancer cells were plated in SSM for 4 h ($3–5 \times 10^4$ per well in a 24-well plate), on plastic, and on hepatocyte-derived ECM; the medium was then changed to HDM, in the presence or absence of 10 ng/ml AR, EGF, or HB-EGF (Sigma). The cells were trypsinized with 0.5% trypsin and 2 mM EDTA at various time points and counted using a hemocytom-

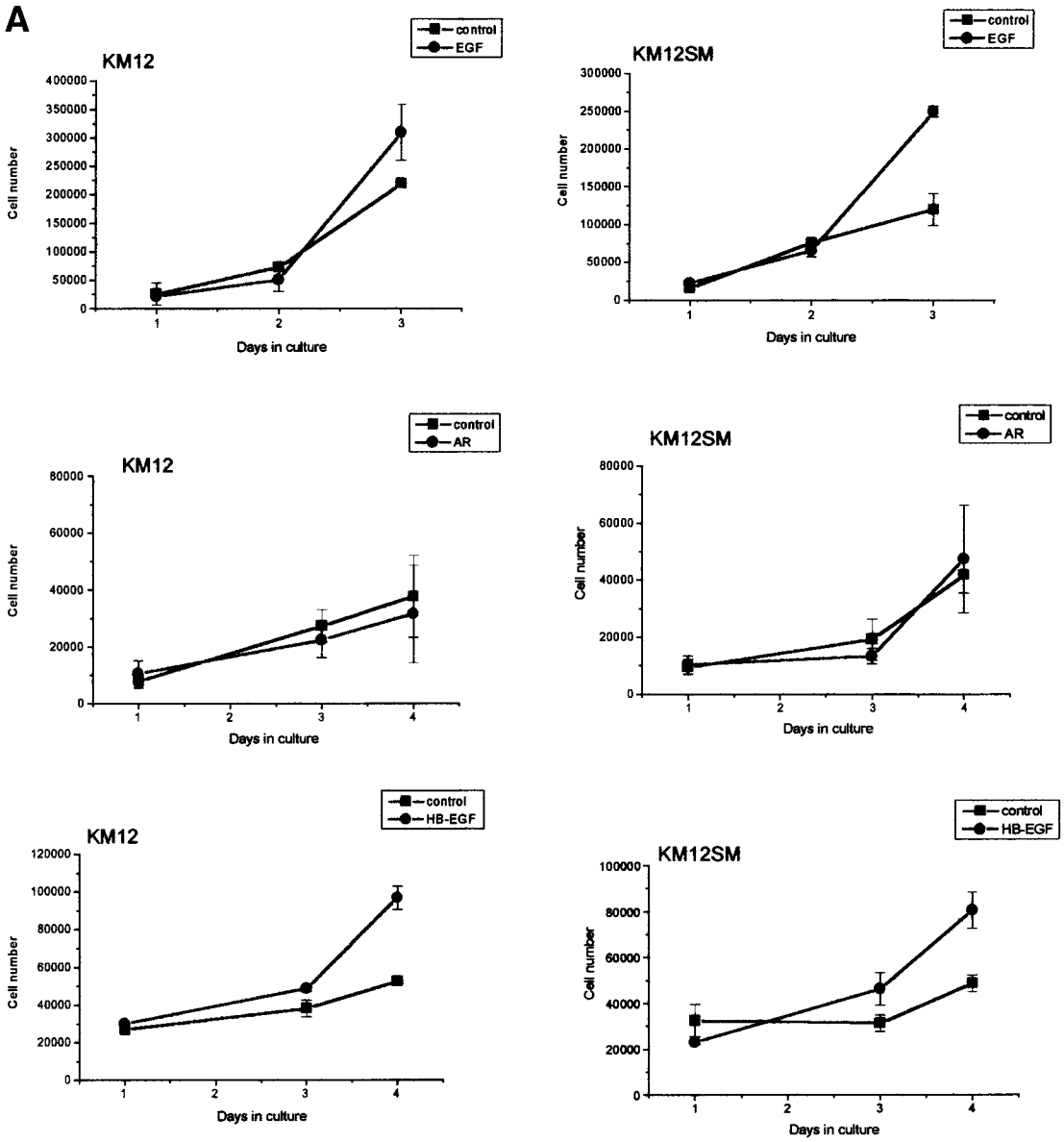

Fig. 1. Growth curves of the weakly metastatic cell line KM12 and the strongly metastatic cell line KM12SM on plastic (**A**) and on hepatocyte-derived extracellular matrix (ECM) (**B**). Cells were cultured as described under Materials and Methods with or without 10 ng/ml epidermal growth factor (EGF), amphiregulin (AR), or HB-EGF. Error bars = standard deviation. For the curves with EGF, HB-EGF, and HB-EGF on ECM, N for each time point = 2; for the AR curves, N = 6; for AR on ECM, N = 4.

eter. Cells were plated in duplicate, and each experiment was repeated at least 3 times.

Statistical calculations were performed using the software program Microcal Origin. We compared the means of control and experimental conditions for each time point, using a *t*-test (two-populations). The results were considered significant at $P < 0.05$. The N value for each point was between 2 and 6. When the counted cell numbers were similar in every experiment, we performed the statistics on the pooled experiments; when the counted cell numbers were dissimilar, we performed the *t*-test on each experiment separately. In each graph, the error bars represent the standard error for the mean of 2–6 counts.

Northern Blot Analysis

Colon cancer cells were plated in SSM for 4 h on plastic and on hepatocyte-derived ECM, then the medium changed to HDM with or without of 10 ng/ml AR (Sigma). After 2 days of culture,

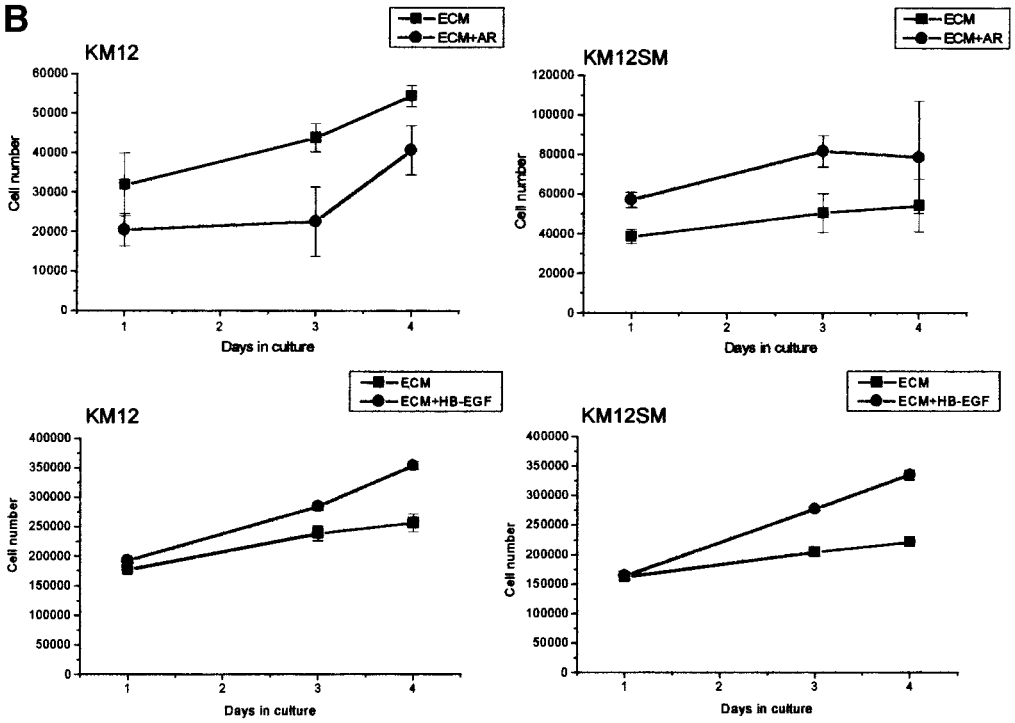

Figure 1. (Continued.)

the cells were washed with cold PBS, and total RNA was isolated from the cells by the method of Chomczynski and Sacchi [1987]. RNA samples were resolved by electrophoresis through 1% agarose and submerged-slab denaturing formaldehyde gels in MOPS buffer and then transferred to Hybond N paper. RNA was fixed to the blots by ultraviolet (UV) crosslinking and then hybridized with cDNA probes radioactively labeled with ^{32}Pd-CTP by primer extension [Feinberg and Vogelstein, 1984]. Blots were hybridized at 37°C for 48 h, washed, and subjected to autoradiography, using X-ray film and enhancing screens. The cDNA probes used were mouse transforming growth factor-α (TGF-α), human AR, and human cripto (Tissue Culture Collection, ATCC).

Western Blots

Colon cancer cells were plated in SSM for 4 h, on plastic and on hepatocyte-derived ECM; the medium then changed to HDM with or without 10 ng/ml AR or HB-EGF (Sigma). Total protein was extracted after 2 days cultures by incubating the cells for 30 min on ice in lysis buffer (250 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris pH 8.0, 0.5% Triton X-100, and 1 mM PMSF); insoluble material was pelleted at 17,000*g*. The extracts were normalized to total protein content, determined using Bradford Reagent (Sigma), and boiled for 5 min in sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol; 5 µg of protein per lane was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (Bio-Rad Protean II minigel) and blotted onto Hybond C extra (Amersham, Arlington Heights, IL). The blots were blocked overnight in Tris-saline buffer pH7.5 and containing 0.05%Tween-20 and 5 milk. Blots were then incubated with antibodies to human erb-B2 (Santa Cruz Biotechnology, Santa Cruz, CA), washed, and incubated for 1 h with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Laboratories, Bar Harbor, ME). The blots were then subjected to chemiluminescence detection and fluororography using X-ray film (NEN Life Science Products, Boston, MA).

Alkaline Phosphatase Assay

Colon cancer cells were plated in 24-well plates and in SSM for 4 h, on plastic and ECM; the medium then changed to HDM with or without 10 ng/ml AR (Sigma). After 2 days in culture, alkaline phosphatase was assayed as described by Sheehan and Hrapchak [1987].

Sites of alkaline phosphatase activity were stained a dark blue-black.

RESULTS

Effect of AR and Hepatocyte ECM on the Proliferation of Colon Cancer Cell Lines

We investigated the effect of three EGF receptor ligands—EGF, AR, and HB-EGF—on the proliferation of the weakly metastatic KM12 and the strongly metastatic KM12SM. All growth factors were used at a concentration of 10 ng/ml, which was found to be optimal for cell proliferation. The growth factors used are recombinant and were used at the concentrations found to be effective by the manufacturers. These concentrations were above the physiological levels of the growth factors in serum or wound fluid, which are within the pg/ml range [Vogt et al., 1998; Yamada et al., 1998]. In solid tissues, however, physiological levels of autocrine and paracrine growth factors are probably much higher than in serum, and their levels would be very difficult to determine.

We found EGF to stimulate the growth of both cell lines, with an effect that was stronger between 2 and 3 days in culture (Fig. 1A). This effect was not significant in KM12 cells at any of the time points but was significant in KM12SM at 4 days ($P < 0.05$).

AR had a slight statistically significant inhibitory effect on the proliferation of KM12 cells and was found to increase the proliferation of KM12SM to a slight but significant extent after 4 days (Fig. 1A). HB-EGF stimulated cell proliferation in both KM12 cells after 3 and 4 days and in KM12SM cells, in which the effect was significant only after 4 days (Fig.1A).

Both AR and HB-EGF are heparin-binding growth factors. We investigated the effect of these growth factors on cells plated on hepatocyte-derived ECM, known to contain heparin proteoglycans. As shown in Figure1B, AR inhibited the growth of the weakly metastatic KM12, but the effect was not significant. At each time point, AR significantly stimulated the proliferation of the strongly metastatic KM12SM. The effect of hepatocyte-derived ECM together with AR was quite specific, since fibroblast-derived ECM together with AR had no effect on the proliferation of the cells (data not shown). HB-EGF significantly stimulated cell proliferation after 3 and 4 days in both KM12 and KM12SM plated on hepatocyte-derived ECM (Fig. 1B).

Effect of AR and Hepatocyte-Derived ECM on the Expression of Autocrine Growth Factors and Their Receptors

In previous studies, we screened for the growth factors and growth factors receptors expressed by KM12 and KM12 SM. We found that the main EGF receptor family members expressed were erb-B2, cripto, and AR [Zvibel et al., 1998a].

The strongest mRNA expression in both cell lines was that of cripto (Fig. 2). Culture on hepatocyte-derived ECM had a different effect on cripto mRNA levels in the colon cell lines: cripto mRNA was lower in KM12 cells and higher in KM12SM. There was no effect of AR on cripto expression in either cell line when the cells were grown on plastic or hepatocyte-derived ECM (Fig. 2). Expression of mRNA for AR was quite weak in both colon cell lines. When cells were cultured on plastic, AR increased mRNA levels for AR in KM12 cells and decreased AR expression in KM12SM (Fig. 2). AR mRNA expression was almost undetectable in both cell lines cultured on hepatocyte-derived ECM (Fig. 2).

TGF-α was present as two low-molecular-weight mRNA species, rather than as the 4.8-kb species usually reported. TGF-α mRNA was decreased in both colon cell lines when they were grown on hepatocyte-derived ECM (Fig. 2).

The effect of AR on erb-B2 expression was different in cells cultured on plastic and on hepatocyte-derived ECM. AR strongly increased erb-B2 levels in cells grown on plastic and reduced erb-B2 expression in cells cultured on ECM (Fig. 3A). HB-EGF increased erb-B2 expression in both KM12 and KM12SM when the cell lines were cultured on plastic (Fig. 3B).

Effect of AR on Differentiation of the Colon Cell Lines

We cultured the cells for 2 days on plastic or on hepatocyte-derived ECM, in the presence or absence of 10 ng/ml AR and tested for the presence of alkaline phosphatase in the colon cell lines cultured in these conditions. KM12 cells strongly expressed alkaline phosphatase, a marker of intestinal epithelial cell differentiation. With ECM, AR or AR+ECM did not alter the expression of this enzyme in KM12 cells. KM12SM showed almost no expression of alkaline phosphatase when the cells were grown on

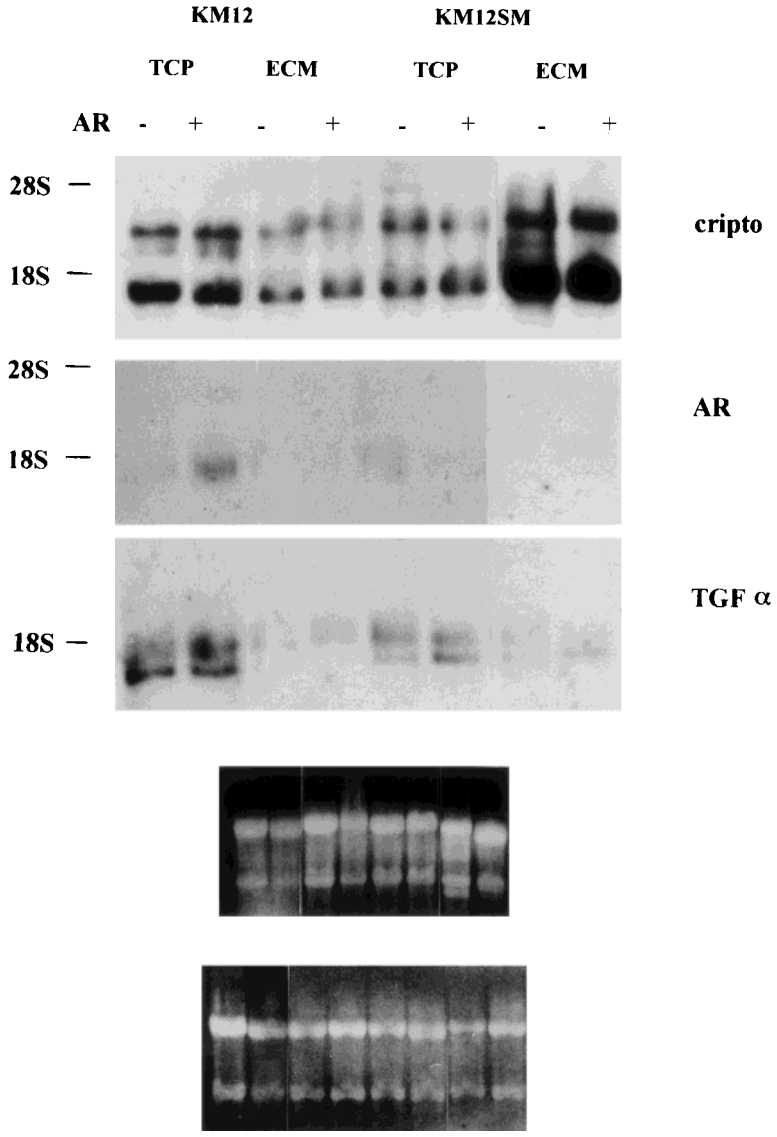

Fig. 2. Northern blot analysis of total RNA from KM12 and KM12SM cells cultured for 2 days on plastic or extracellular matrix (ECM), with or without 10 ng/ml AR. A total of 10 µg of total RNA was loaded per slot; blots were hybridized with ^{32}P-dCTP probes labeled as described under Materials and Methods. Ethidium bromide (**top**) represents the upper two Northern blots; ethidium bromide (**bottom**) represents the lowest Northern blot and shows loading.

plastic, but extremely low expression was observed in about 10% of the cells when the cells were grown on hepatocyte-derived ECM. AR did not alter this expression.

DISCUSSION

We have previously shown that hepatocyte-derived ECM stimulated the proliferation of colon cancer cell lines of varying liver colonizing potential. In the same studies, we found that hepatocyte ECM enhanced expression of erb-B2 and cripto but did not affect the expression of the EGF receptor or AR. By modifying the ECM produced by hepatocytes, we showed that at least some of its effects were attributable to heparin proteoglycans [Zvibel et al., 1998a].

We were interested in determining the effect of the autocrine growth factors expressed by the colon cell lines in conjunction with hepatocyte ECM. The local milieu of the colonized organ plays a major role in the response of the colonizing tumor cells to both autocrine and paracrine growth factors.

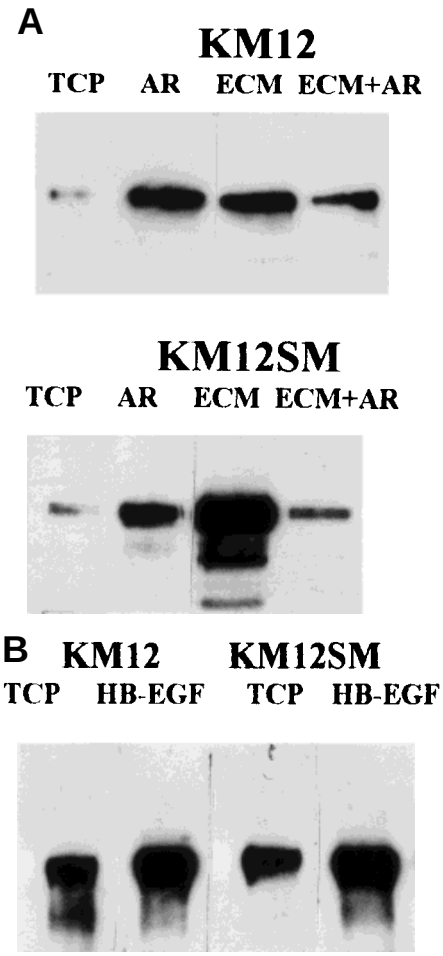

Fig. 3. Western blots showing erb-B2 expression in KM12 and KM12SM cell lines cultured for 2 days on plastic (TCP) or ECM with or without 10 ng/ml AR (**A**) and on plastic (TCP) with or without 10 ng/ml HB-EGF (**B**). A total of 5 µg of total protein was loaded per lane.

Site-specific metastasis is known to be determined by several properties of both tumor cells and host [Nicolson, 1989]. The target organ regulates the growth of the tumor cells either by secreted soluble growth factors produced by the cells of the organ or by growth factors embedded in the local ECM [Nicolson and Dulski, 1986; Zvibel and Kraft, 1993]. Alternatively, the local specific ECM of the target organ can affect the proliferation of the tumor cells [Doerr et al., 1989; Zvibel and Kraft, 1993]. We have previously reported that the ability of tumor cells to metastasize in vivo to certain organs correlates with the ability of the cells to survive on ECM derived from that organ [Doerr et al., 1989; Kraft et al., 1992]. We found that the ECM components responsible for this effect were heparin proteoglycans and that they acted via regulation of autocrine growth factors expression in the tumor cells [Zvibel et al., 1991].

The EGF receptor family, crucial in colon tumors [Ciardello et al., 1991], consists of four known receptors: EGF receptor, or erb-B1, erb-B2, erb-B3, and erb-B4 [Beerli and Hynes, 1996]. These receptors can form either homodimers or heterodimers with each other and can bind and respond to different ligands, such as EGF, TGF-α, HB-EGF, betacellulin, AR, and the heregulins, according to the receptors present in the complex. Another member of the EGF family is cripto.

In the present studies, we found a stimulatory response to HB-EGF in both colon cancer cell lines cultured on plastic. However, AR inhibited the growth of the weakly metastatic cells KM12. Both AR and EGF had a stimulatory on the proliferation of the strongly metastatic cells KM12SM. Low response of cells to AR was shown to correlate with low numbers of EGF receptors [Adam et al., 1996]. AR is able to induce phosphorylation of both EGF receptor and erb-B2; however, it acts exclusively through the EGF receptor [Johnson et al., 1993]. By contrast, HB-EGF binds to both EGF receptor and erb-B4 [Elenius et al., 1997]. Thus, responsiveness or lack of responsiveness to AR or HB-EGF could be dictated by the number of EGF receptors, by the presence of other EGF receptors, as well as by the presence of heparan sulfate. We have shown that KM12 and KM12SM cells express EGF receptors, erb-B2, and erb-B3, in similar amounts, except that erb-B3 is present in lower amounts in KM12 cells [Zvibel et al., 1998a,b, and data not shown]. The expression of both erb-B2 and erb-B3 was increased in both colon cell lines when the cells were cultured on hepatocyte-derived ECM. Expression of erb-B4 receptors remains to be tested.

One of the elements modifying the response of cells to a growth factor is the ECM surrounding the cell. The ECM of the normal liver is found in the space of Disse and is produced by endothelial cells, Ito cells, Kupffer cells, and hepatocytes. The hepatic ECM consists mainly of fibrillar collagen types I and III, aggregates of type IV and fibronectin [Martinez-Hernandez and Amenta, 1993]. The main hepatocyte proteoglycan is syndecan-2 or fibroglycan [Lyon and Gallagher, 1991], but hepatocytes also express syndecans-1 and -4 [Kim et al., 1994]. The glycosaminoglycan chains of these syndecans

are unique to the liver and are chemically very similar to the heparin produced by mast cells [Lyon et al., 1994].

AR and HB-EGF are heparin-binding growth factors, and their effects on a given cell were shown to be modified in the presence of heparin [Johnson and Wong, 1994; Cook et al., 1995]. AR, but not HB-EGF, requires cell surface heparin proteoglycans for mitogenic activity [Johnson and Wong, 1994; Cook et al., 1995].

When the colon cells were plated on hepatocyte-derived ECM, both AR and HB-EGF evoked a response in the cells. AR inhibited the growth of the weakly liver-colonizing KM12 cell line (although not in a statistically significant manner), while the strongly metastatic cells KM12SM were stimulated to proliferate. HB-EGF stimulated proliferation of both cell lines. This finding suggests a possible mechanism for the proliferation of colorectal tumor cells in the liver, with highly metastatic liver-colonizing colon cells being stimulated to proliferate by the synergistic signal of autocrine growth factors and the surrounding hepatic ECM.

Ligands for EGF receptor lead to autoinduction of various other members of the EGF family in human keratinocytes; thus, EGF and TGF-α for example, induce both auto expression as well as expression of HB-EGF and AR [Stoll et al., 1997; Barnard et al., 1994]. The mechanism is attributable to stabilization of the mRNA for the growth factors. We found that both AR and HB-EGF increased erb-B2 expression in the two colon cancer cell lines grown on plastic, and AR decreased erb-B2 expression in both cell lines grown on ECM. No correlation was found between cell proliferation and erb-B2 expression. Erb-B2 is probably not the sole mediator of cell proliferation in these cells.

AR did not increase mRNA expression for TGF-α and cripto but did increase AR expression in KM12 cells grown on plastic. Since AR was inhibitory for the weakly metastatic KM12 and stimulatory for the strongly metastatic KM12SM, the increase of AR in KM12 cells could result in cell inhibition. Cells of the normal intestine were shown to respond differently to growth factors, depending on their differentiation state. TGF-α stimulated stem cells at the base of the crypt but, as they moved toward the tip of the villus and differentiated, both goblet cells and enterocytes no longer responded to TGF-α with a proliferative response [Huang et al., 1995]. We tested the role of AR in cell differentiation of the two colon cell lines but did not find a significant effect as measured by alkaline phosphatase expression.

The final fate of the colon cancer cell metastasizing to the liver will be determined by the interplay between the stimulatory effect of the ECM, the effect of the cell-derived soluble factors, and the response of the colon cancer cells. This response is dictated by the state of differentiation of the colon cell, by the autocrine growth factors and receptors expressed, and by the chemistry of the surrounding ECM.

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