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Cell Biology

Genetics and biochemistry of collagen binding-triggered glandular differentiation in a human colon carcinoma cell line

[attachment/Arg-Gly-Asp (RGD)/receptor/hybrids/chromosome 15]

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ABSTRACT **We have examined the interaction between collagen binding and epithelial differentiation by using a human colon carcinoma cell line (SW1222) that can differentiate structurally when grown in a three-dimensional collagen gel to form glandular structures. As much as 66% inhibition of glandular differentiation can be achieved by addition to the culture of a synthetic peptide (2 mg/ml) containing the Arg-Gly-Asp-Thr (RGDT) sequence, which is a cell recognition site found in collagen. Arg-Gly-Asp-Thr also inhibited the cell attachment to collagen-coated plates. A control peptide containing the Arg-Gly-Glu-Thr (RGET) sequence had no effect on cell adhesion or cell differentiation. Chromosome 15 was found in all human–mouse hybrid clones [from a cross between SW1222 and a mouse rectal carcinoma cell line (CMT-93)] that could differentiate in the collagen gel and bind collagen. Both binding to collagen and glandular differentiation of the hybrid cells were also inhibited by Arg-Gly-Asp-Thr as for the parent cell line SW1222. The ability of SW1222 cells to express the differentiated phenotype appears, therefore, to be determined by an Arg-Gly-Asp-directed collagen receptor on the cell surface that is controlled by a gene on chromosome 15.**

Collagens can influence the embryological development of epithelial cells and their ability to maintain or to re-express the differentiated phenotype, cell polarity, and morphological organization (1–3). However, the molecular events controlling epithelial cell–collagen interactions and their consequences have not yet been characterized. It has been proposed that either (*i*) binding to collagen may induce a cell to become more responsive to soluble factors produced by mesenchymal or epithelial cells or (*ii*) that the collagen itself may trigger a variety of changes in cell behavior (4).

Fibronectin and laminin have been shown to mediate the attachment of various cell types to collagens type I and IV, respectively (5). Some cell types also bind collagen directly (6), which suggests the presence of specific cell-surface receptors for collagen other than fibronectin or laminin. Pierschbacher and co-workers identified an amino acid sequence, Arg-Gly-Asp (RGD), as an essential structure mediating cellular binding to fibronectin (7), vitronectin (8), and type I collagen (9), and these results have enabled the isolation of their specific receptors (10). However, it is not yet known whether these receptors may also mediate changes in differentiation by acting as transducers of signals between the extracellular matrix and the cytoskeleton. Human carcinoma-derived cell lines that can re-express some aspects of the normal differentiated phenotype in response to the extracellular matrix are useful tools for studying epithelial cell–extracellular matrix interactions.

SW1222 is a human colon carcinoma-derived cell line that expresses *in vitro* typical epithelial polarity and glandular organization, as in normal intestinal glands, but this expression occurs only when cells are grown embedded in a three-dimensional (3D) collagen gel (11). Other similar cell lines tested did not show this ability. This difference suggests that SW1222 cells retain the ability to interact with collagen, probably through a specific collagen receptor, with the subsequent triggering of molecular events that lead to structural glandular differentiation.

Therefore, we have investigated whether there is an RGD receptor on SW1222 cells that mediates cellular attachment to collagen and, subsequently, the differentiated phenotype seen in 3D collagen gels. Furthermore, to map the putative gene(s) controlling collagen binding and morphological differentiation we produced a panel of somatic cell hybrids by fusing SW1222 cells with a mouse rectal carcinoma-derived cell line (CMT-93) that shows no glandular organization in a collagen gel. The results suggest that the differentiation of SW1222 in collagen gels is mediated by an RGD collagen receptor controlled by a gene on chromosome 15.

MATERIALS AND METHODS

Cells. The SW1222, SW620, and LS174T human colorectal carcinoma-derived cell lines (12, 13) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in 10% CO_2. CMT-93, an 8-azaguanine-resistant variant of a mouse rectal carcinoma-derived cell line (14) was maintained in DMEM containing 10% fetal calf serum and 2×10^{-5} M 6-thioguanine at 37°C in 10% CO_2.

Production of the Hybrid Cells (SWC Series). Somatic cell hybridization was done according to Galfre *et al.* (15). SW1222 cells (5×10^6) were fused with an equal number of the mouse CMT-93 cells (hypoxanthine-guanine phosphoribosyltransferase variant) using polyethylene glycol 1500 (PEG, BDH Chemicals) (50% wt/vol in DMEM) (16). Then fused cells were plated at low density in DMEM/20% fetal calf serum with 10^{-4} M hypoxanthine/1.6×10^{-5} M thymidine/10^{-5} M methotrexate (HAT) and 1 mM ouabain (Sigma). Individual hybrid colonies were picked after 14 days, and one of them (SWC 3) was subsequently recloned. From 24 initial colonies and derived clones, 15 hybrids (SWC 2, 3, 7, 6A, 6B, 2D, 2E, 3/3, 3/4, 3/5, 3/6, 3/7, 3/8, 3/9, 3/10), containing a variable number of human chromosomes, were further evaluated for their ability to express the differentiated phenotype in collagen gels.

Human Chromosome and Marker Analysis. The human chromosomes present in the hybrids were identified by isozyme and karyotype analysis; isozyme analysis was accomplished using starch gel or cellulose gel electrophoresis and standard techniques (17). Karyotyping was carried out using G-11 banding (18), Q-banding (19), and G-banding (20)

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Abbreviations: RGD peptide, Arg-Gly-Asp; 3D, three-dimensional; mAb, monoclonal antibody.

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techniques. In addition the hybrids were typed with a panel of monoclonal antibodies (mAbs) recognizing human cell-surface determinants using an indirect immunoperoxidase technique (21). The panel of mAbs and the chromosomes to which their reactions map were as follows: mAb TRA -2-10 (chromosome 1) (ref. 22), mAb AUA1 (chromosome 2) (ref. 23), mAb OKT9 (chromosome 3) (ref. 24), mAb W6/32 (chromosome 6) (ref. 25), mAb EGFR 1 (chromosome 7) (ref. 26), mAb 163A5 (chromosome 11) (ref. 27), mAb 602 (chromosome 12) (ref. 28), mAb BBM1 (chromosome 15) (ref. 29), mAb H207 (chromosome 17) (ref. 30), and mAb 12E7 (chromosomes X and Y) (ref. 31).

Collagen Gel Preparation. Collagen gels were prepared using Vitrogen 100 collagen (Collagen, Palo Alto, CA) according to the manufacturer's instructions. Vitrogen 100 collagen is 99.9% pure collagen as judged by $NaDodSO_4$ polyacrylamide gel electrophoresis in conjunction with bacterial collagenase sensitivity and silver staining techniques. It is 95–98% type I collagen with the remainder being type III collagen. Cells (10^5) were mixed with 1 ml of the neutralized Vitrogen 100 collagen solution (pH 7.4–0.2) and plated into 24-well tissue culture plates kept at 4°C (Falcon 3047, Becton Dickinson). In some experiments the cells were resuspended in 50 μl of DMEM plus bovine serum albumin at 2.5 mg/ml and 25 mM Hepes (pH 7.4) containing Ser-Arg-Gly-Asp-Thr-Gly or Ser-Arg-Gly-Glu-Thr-Gly (2 mg/ml) (provided by J. Rothbard, Imperial Cancer Research Fund, London) and then mixed with the neutralized collagen solution as described above. Collagen gelation was then initiated by warming the collagen solution to 37°C for 10–20 min. The gel was then overlaid with 1 ml of DMEM/10% fetal calf serum medium, which was changed twice a week. After 14 days collagen gels were fixed with neutral buffered formalin for 24 hr, removed from the wells, and embedded in paraffin wax. Cross sections were then cut from paraffin blocks and stained with hematoxylin/eosin (11).

Collagen Binding Assay. Microtiter plates (Dynatech, New York) were coated with a thin layer (25 μl/well) of either bovine serum albumin or Vitrogen 100 solution (20 μg/ml) and left uncovered in a laminar flow hood overnight to allow normal evaporation. The plates were then rinsed with DMEM and used in the binding assay. Trypsinized cells were washed three times in DMEM containing bovine serum albumin at 2.5 mg/ml and 25 mM Hepes (pH 7.4). Approximately 2×10^4 cells per well were plated into 96-well Dynatech plates previously coated. Cells were allowed to attach for 3 hr at 17°C as described by Dedhar *et al.* (9). Ser-Arg-Gly-Asp-Thr-Gly, Ser-Arg-Gly-Glu-Thr-Gly, Gly-Arg-Gly-Asp-Ser-Pro, and Gly-Arg-Gly-Glu-Ser-Pro (synthetic peptides) were added to some of the wells at concentrations ranging from 0.125 to 2 mg per ml. The supernatants were removed, and the unattached cells were washed away three times with DMEM. The numbers of attached cells were estimated by measuring alkaline phosphatase activity (32). Briefly, 1.2 mM 4-methylumbelliferyl phosphate was added to the wells in a buffer containing 0.2 M boric acid and 1 mM $MgCl_2$. This substrate reacts with alkaline phosphatase to produce free phosphate and 5-methylumbelliferylerone, a highly fluorigenic compound. The fluorescence intensity measured on a Dynatech microfluor reader and expressed in arbitrary units is then proportional to the numbers of cells remaining in the well.

RESULTS

Pattern of Growth of SW1222 Cells Under Standard Conditions and in 3D Collagen Gels. SW1222 cells grew as tightly packed clumps, exhibiting a typical epithelial morphology but without any glandular organization. When the cells were resuspended in a 3D collagen gel a clear glandular differentiation pattern was seen, as already described (11). Sections through paraffin-embedded gels showed a striking similarity between these structures and normal colonic crypts. Many of the cells were columnar-like with nuclei polarized to the periphery, while interspersed signet ring-like cells were also present (Fig. 1). This structural organization has been confirmed by electron microscopy (11). A semi-quantitative analysis of the grade of differentiation, based on counting the glandular structures in a field, showed a progressive increase with time in the number of glandular structures per total number of cell colonies. During culture in the collagen gel this number reached a plateau at about 7–10 days of 32.5% (see Fig. 3*B*). Morphological differentiation was accompanied by inhibition of growth (data not shown). When the cells were resuspended in an agarose gel, no glandular differentiation was seen.

Effect of RGD-Containing Peptides on Collagen Binding and Glandular Differentiation of SW1222 Cells. SW1222 cells bound directly and specifically to collagen-coated plates, as shown in Fig. 2*A*. Two other colon carcinoma-derived cell lines, LS 174T and SW620, which did not form organized structures in 3D collagen gels, lacked this ability to bind collagen. Peptides Ser-Arg-Gly-Asp-Thr-Gly (RGDT) and Gly-Arg-Gly-Asp-Ser-Pro (RGDS) both inhibited SW1222 collagen binding; the former was more effective (50% inhibition of binding at 0.75 mg/ml concentration) than the latter (Fig. 3*A*). The two control peptides, Ser-Arg-Gly-Glu-Thr-Gly (RGET) and Gly-Arg-Gly-Glu-Ser-Pro (RGES) had no effect on collagen binding. The addition of Ser-Arg-Gly-Asp-Thr-Gly (2 mg/ml) to SW1222 cells growing in a 3D collagen gel markedly decreased the degree of glandular differentiation and the number of clusters, giving rise to a much higher proportion of single cells. The maximum proportion of glandular structures/colony of cells was significantly decreased in cultures containing Ser-Arg-Gly-Asp-Thr-Gly (10%) as compared with the cell cultures containing either no peptide (32%) or the inactive Ser-Arg-Gly-Glu-Thr-Gly (30%) (Fig. 3*B*).

Pattern of Growth of the SWC (SW1222 × CM-T93) Hybrids Under Standard Conditions and in 3D Collagen Gels. All SWC hybrids showed undifferentiated patterns of growth

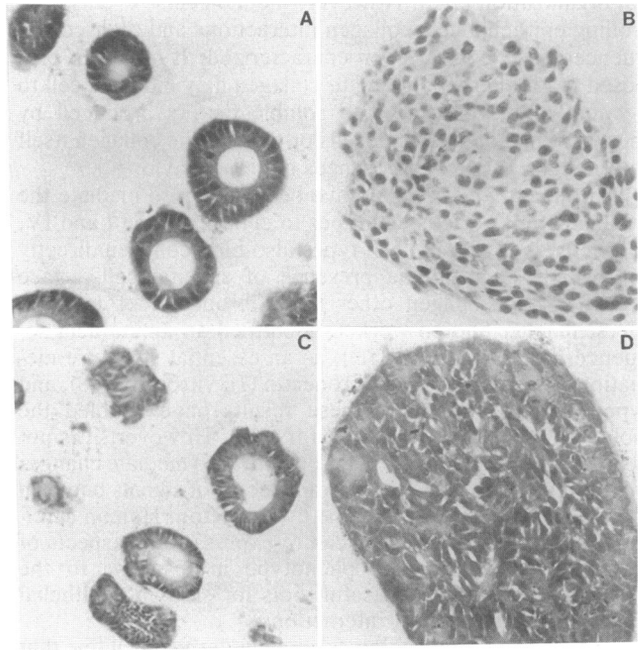

FIG. 1. SW1222 (*A*), CMT-93 (*B*), SWC 3 (*C*), and SWC 2 (*D*) parent cells and hybrids in collagen gel. SW1222 and SWC 3 cells are organized around a central lumen with nuclei polarized to the periphery. CMT-93 and SWC 2 show an undifferentiated pattern of growth. (Hematoxylin/eosin; ×66.)

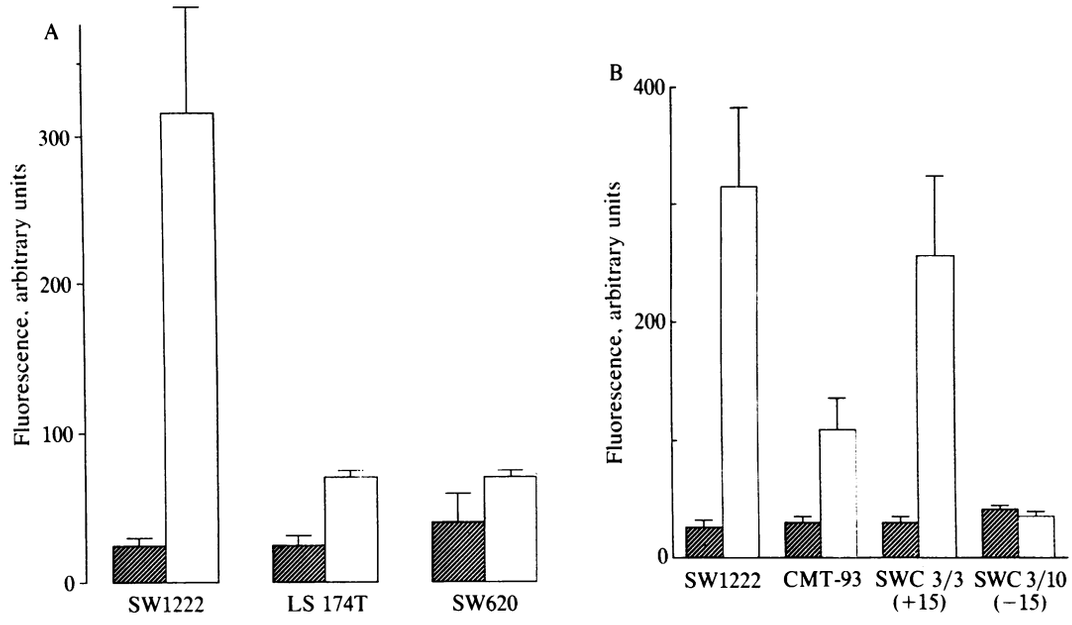

FIG. 2. Collagen binding of SW1222, LS 174T, SW620 (*A*), and SW1222, CMT-93, and SWC 3 hybrid clones 3 (containing chromosome 15) and 10 (minus chromosome 15) (*B*). Clear columns represent the cell binding to collagen-coated (20 μg/ml) plates, and the hatched columns represent binding to bovine serum albumin (20 μg/ml). Data shown represent the mean ± SD of three determinations.

in Petri dishes, as did the parent cells SW1222 and CMT-93, and all had typical epithelial structure. Two of seven primary hybrid colonies formed differentiated glandular structures like the human parent cell line (SW1222) when grown in 3D collagen gels (Fig. 1 *A* and *C*), whereas the remainder did not, following the mouse parent CMT-93 (Fig. 1 *B* and *D*). Subclones from one of the hybrids SWC3 also segregated (3:5) for this ability to differentiate. However the number of glandular structures in the hybrid clones was less (12%) than that in the human parent cell line SW1222 (21%). A combined analysis of karyotypes, isoenzymes, and mAb immunostaining of these hybrids showed that only chromosome 15 segregated with the structural differentiation in collagen gels (Table 1).

Effect of RGD-Containing Peptides on Collagen Binding and Glandular Differentiation of SWC Hybrids. As shown in Fig. 2*B*, a hybrid containing chromosome 15 (SWC 3/3) showed high levels of collagen binding in contrast to the hybrid (SWC 3/10) that lacked chromosome 15 and did not bind collagen. In this experiment CMT-93 showed some collagen binding but clearly much less than did SW1222 or SWC 3/3. Binding to collagen-coated plates was inhibited by the Ser-Arg-Gly-Asp-Thr-Gly (2 mg/ml), as for the human parent cell line SW1222 (Fig. 4). In addition, the number of glandular structures per colony of cells in the 3D collagen gel was also significantly reduced by the Ser-Arg-Gly-Asp-Thr-Gly (RGDT) (3%) as compared with the inactive Ser-Arg-Gly-Glu-Thr-Gly (RGET) (12%).

DISCUSSION

In this study we examined the relationship between collagen binding and structural differentiation of the human colon carcinoma cell line SW1222 and a panel of derived hybrids. We showed that SW1222 cells can bind collagen directly and form crypt-like structures in 3D collagen gels, whereas two

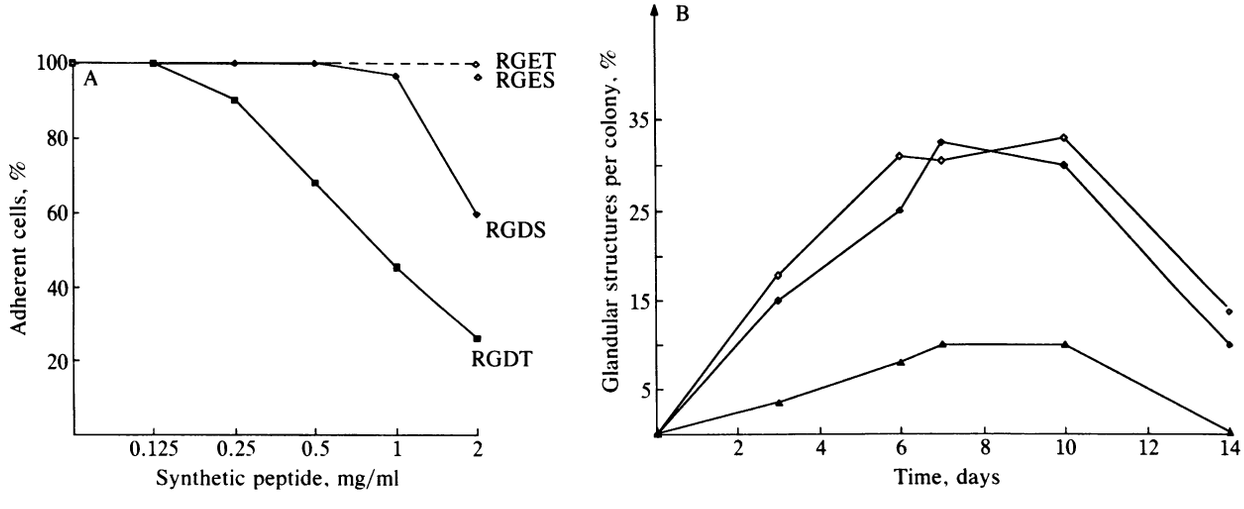

FIG. 3. (*A*) Effect of Ser-Arg-Gly-Asp-Thr-Gly (RGDT), Ser-Arg-Gly-Glu-Thr-Gly (RGET), Gly-Arg-Gly-Asp-Ser-Pro (RGDS), and Gly-Arg-Gly-Glu-Ser-Pro (RGES) on collagen binding of SW1222 cells. (*B*) Effect of Ser-Arg-Gly-Asp-Thr-Gly (▲) and Ser-Arg-Gly-Glu-Thr-Gly (□) (2 mg/ml) on glandular differentiation in SW1222 cells. ■, Negative control, where no peptide was added. Two hundred colonies were counted, and the glandular structures were identified under a phase-contrast Diavert Leitz microscope (objective 32L) at days 3, 6, 7, 10, and 14. Values are expressed as percentage of the number of glandular structures per number of cell colonies.

Table 1. Segregation of structural differentiation of SWC hybrids with chromosome 15

Parents and hybrids	Chromosome																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Diff.
SW1222	+	+	+	+	+	+	+	+	+	+	+	+	+	+	**+**	+	+	+	+	+	+	+	+	+
CMT-93	−	−	−	−	−	−	−	−	−	−	−	−	−	−	**−**	−	−	−	−	−	−	−	−	−
SWC 6B	−	+				+	−				+	−			**+**		−		−				+	+
SWC 3	+	−	+	−	−	−	−	−	−	−	−	−	+	+	**+**	−	−	−	−	−	+	+	+	+
SWC 3/3	−	−	−	−	−	−	−	−	−	−	−	−	+	−	**+**	−	−	−	−	−	+	+	+	+
SWC 3/5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	**+**	−	−	−	−	−	−	+	+	+
SWC 3/7	−	−	−	−	−	−	−	−	−	−	−	−	+	−	**+**	−	−	−	−	−	+	+	+	+
SWC 2	−	−	+	+	−	+	+	−	−	−	−	−	−	−	**−**	−	−	−	−	−	−	−	+	−
SWC 6A	+	−	−	−	−	−	+	−	+	−	+	+	+	−	**−**	−	−	+	−	−	+	+	+	−
SWC 2E	+	−			−	−					+	+			**−**		+		+				+	−
SWC 2D	−	−	+	−	+	+	+	−	−	+	−	−	−	−	**−**	−	−	+	−	−	+	+	+	−
SWC 7	+	−	+	−	−	+	−	−	−	−	+	−	+	+	**−**	−	+	−	−	−	+	+	+	−
SWC 3/4	−	−	+	−	−	−	−	−	−	−	−	−	−	−	**−**	−	−	−	−	−	−	+	+	−
SWC 3/6	−	−	+	−	−	−	−	−	−	−	−	+	+	−	**−**	−	−	−	−	−	+	+	+	−
SWC 3/8	−	−	+	−	−	−	−	−	−	−	−	−	−	−	**−**	−	−	−	−	−	+	+	+	−
SWC 3/9	−	−	+	−	−	−	−	−	−	−	−	−	+	−	**−**	−	−	−	−	−	+	+	+	−
SWC 3/10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	**−**	−	−	−	−	−	+	+	+	−

SWC 6B, 3, 2, 6A, 2E, 2D, and 7 are primary hybrids. SWC 3/3–10 are subclones of SWC 3. Chromosome assignment (+, −) was based on a combination of karyotype, antibody, and enzyme analysis. Chromosome 15 segregation (boldface) was most clearly established by reactivity with BBM1, a mAb against human β_2-microglobulin. Diff. (+, −) indicates the ability to differentiate and form glandular structures in collagen gels.

other cell lines, LS 174T and SW620, neither bind collagen nor can differentiate in collagen gels. We also showed a similar relationship between collagen binding and glandular differentiation among the SWC human–mouse (SW1222 × CMT-93) hybrids that contain human chromosome 15. These findings suggest that the ability of SW1222 cells to express the differentiated phenotype is determined by the presence of a collagen receptor on the cell surface and that this receptor is controlled by a gene on chromosome 15.

The RGD sequence is present in four different locations in the α_2 chain and in two locations in the α_1 chain of the triple helical region of type I collagen (33). Recently Dedhar *et al.* (9) identified a type I collagen receptor complex on a human osteosarcoma cell line, MG-63, recognizing the RGD sequence. We have shown that the SW1222 cell collagen binding is also mediated by an RGD-directed receptor. Ser-Arg-Gly-Asp-Thr-Gly caused 75% inhibition of cell attachment when it was added in soluble form to the collagen. Furthermore, addition of the same Ser-Arg-Gly-Asp-Thr-Gly to SW1222 cells growing in 3D collagen gels significantly decreased glandular differentiation. When the aspartic acid (D) was substituted by a glutamic residue (E) in the peptide, no inhibitory effect on either cell attachment or glandular differentiation was seen. Therefore, we believe that the RGD sequence is an essential recognition signal for the binding of SW1222 to collagen and that this binding triggers glandular differentiation. Two lines of evidence suggest that the receptor we have identified in SW1222 may be specific for collagen type I. (*i*) The preparation used to make the collagen gels is >95% collagen type I. (*ii*) The greater inhibiting activity of Ser-Arg-Gly-Asp-Thr-Gly as compared with Gly-Arg-Gly-Asp-Ser-Pro agrees with the specificity of the collagen type I receptor described by Dedhar *et al.* (9). In addition preliminary data show agreement between binding of I^{125}-labeled collagen (Vitrogen 100) and attachment of cells to collagen-coated plates and 3D collagen gel-induced differentiation. That the binding is due to fibronectin seems unlikely because this binding is known to be highly trypsin sensitive and does not map to chromosome 15 (34, 35). Furthermore, using indirect immunoperoxidase staining, mAb FN-3, which

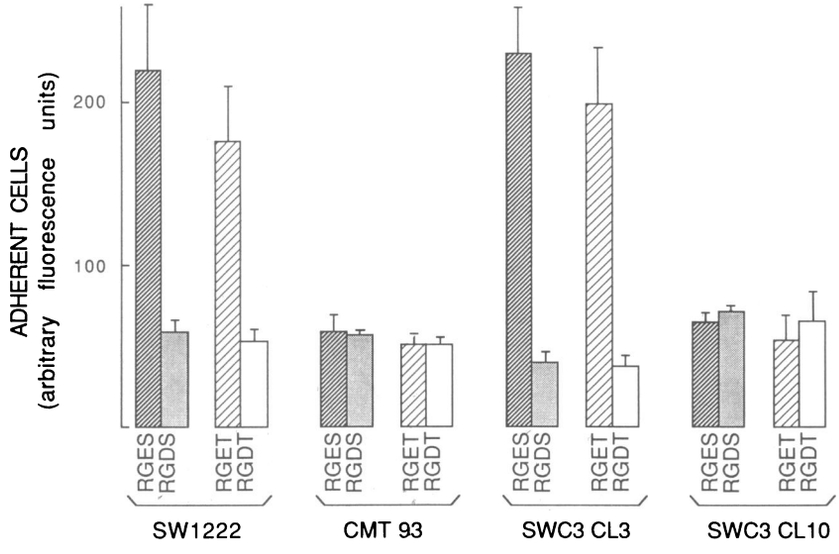

FIG. 4. Effect of RGD-containing peptides (2 mg/ml) on collagen binding of SWC 3 clones 3 and 10 hybrids. For peptide identification see Fig. 3 legend.

recognizes cell-associated fibronectin (36), did not react with the cell surface of SW1222 cells. Further studies are needed to characterize the nature of this receptor and to establish whether, as might be expected, the pericryptal smooth muscle cells are the source of type I collagen, which stimulates the differentiation of cryptal epithelial cells *in vivo* (37).

The genetic mapping and characterization of the gene(s) controlling collagen binding and structural differentiation was studied using interspecific somatic cell hybrids between SW1222 and a mouse rectal carcinoma-derived cell line, CMT-93. The mouse cell line was chosen to avoid the problem of extinction that could follow from the use of a parent cell of heterologous tissue type, and this cell line shows no glandular organization when cultured in 3D collagen gels (Fig. 1*B*). Somatic cell hybrids are useful tools for studying the genetic control of cell differentiation (38). Hybrid clones containing a restricted number of human chromosomes can be analyzed for their ability to express the differentiated phenotype of the human parent cell line. Using this approach we showed that the gene(s) controlling both collagen binding and glandular differentiation maps to chromosome 15, and this localization provides a powerful adjunct for characterizing the corresponding receptor. The study of hybrids with a nonepithelial mouse parent is needed to establish tissue specificity of this control. The data suggest that genetic control of this differentiation is dominant and explicable by the presence of a cell-adhesion receptor on SW1222 that recognizes the RGD sequence in collagen. This receptor plays a critical role in triggering the glandular differentiation seen in 3D collagen gels, presumably via a stimulus through attachment of collagen to its cell-surface receptor. This triggering may well occur by similar signaling processes to those used by soluble growth factors and their receptors.

The pathologist's definition of tumor differentiation relates largely to structure and architecture. This aspect of differentiation is one of the most important factors affecting prognosis in colorectal carcinoma (39). Differentiation of SW1222 cells in 3D collagen gels may, therefore, provide a good model for studying this aspect of differentiation. Escape from this control of differentiation, which must limit the growth potential of a developing tumor, is probably strongly favored by selection. Such selection would be for loss of ability to synthesize the collagen receptor and would thus be one of the class of recessive genetic changes that has been discovered recently using genetic marker studies (40–42).

In general, direct expression of receptors for differentiation (such as for extracellular matrix components or soluble factors) and their ligands, as well as positive regulators for such expression, may explain a major class of recessively acting oncogenes. This explanation follows from the general complimentarity between growth and differentiation and is consistent with the low proportion of colon-carcinoma-derived cell lines that we have found to express the collagen receptor. Similar mechanisms may also be involved in the somatic evolution of metastases during tumor progression. These mechanisms compliment those connected with the dominantly acting oncogenes involved in positive growth control (43). These ideas are beginning to provide a general functional framework for the analysis of the steps in tumor progression, which now can be studied at both the genetic and biochemical levels.

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