Thrombospondin-induced Adhesion of Human Keratinocytes

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Abstract

Human epidermal keratinocytes obtained from normal skin attached and spread on thrombospondin (TSP)-coated plastic dishes but failed to attach and spread on untreated plastic culture dishes or dishes coated with fibronectin or laminin. These cells produced minimal amounts of immunoreactive TSP. Keratinocytes established in culture on MCDB 153 medium and maintained for one to three passages in an undifferentiated state by continued cultivation in this low Ca^{2+}-containing medium attached and spread on plastic dishes as well as on TSP-coated dishes. These cells also secreted significant amounts of TSP into the culture medium. When the keratinocytes were incubated for one day in MCDB 153 medium supplemented with high Ca^{2+} or in MEM (which also contains high Ca^{2+}), there was decreased secretion of TSP into the culture medium concomitant with a reduction in attachment and spreading on plastic culture dishes.

Proteolytic fragments of TSP were examined for stimulation of keratinocyte attachment and spreading. A 140-kd fragment produced by removal of the 25-kd heparin-binding domain had similar activity to the intact molecule while the 25-kd fragment was without effect. Further proteolytic treatment of the 140-kd fragment gave rise to a fragment consisting of 120 kd and 18-D moieties held together in disulphide linkage. This fragment did not support attachment or spreading.

This study reveals that normal epidermal keratinocytes grown under conditions that maintain the undifferentiated state are able to produce TSP and utilize it as an attachment factor. When keratinocytes are grown under conditions that promote differentiation, ability to produce and utilize TSP is diminished. Since TSP is present at the dermal-epidermal junction and because TSP promotes keratinocyte attachment and spreading, this molecule may play an important role in maintaining normal growth of the basal cell layer and may also participate in reepithelialization during wound repair.

Introduction

Thrombospondin (TSP)[1] is a high molecular weight glycoprotein component of platelet α granules (1, 2). It is released from platelets during activation and participates in the secondary phase of platelet aggregation (3). Recent evidence from a number of investigators suggests that in addition to its role in platelet function, TSP may have a much more diverse physiological role. TSP is synthesized by a variety of different cell types and incorporated into the extracellular matrix of these cells (4–11). Like other components of the extracellular matrix, TSP stimulates cell attachment and spreading (8, 10, 12).

Studies from our laboratory have focused on the role of TSP in squamous carcinoma cell behavior. Our studies have shown that TSP mediates attachment and spreading of these cells (10). Among a series of squamous carcinoma cell lines, there was a direct relationship between amount of TSP synthesized and secreted into the culture medium and the adhesiveness of the cells under basal conditions (i.e., on plastic dishes in the presence of BSA) or on TSP-coated dishes. Additionally, it was shown that polyclonal or monoclonal antibodies to TSP inhibited basal adhesion as well as TSP-induced attachment and spreading. TSP may be particularly important as an adhesion factor for squamous carcinoma cells since these cells are less responsive than other tumor cells to laminin or fibronectin (10, 13). In the present study, we have examined the possible involvement of TSP as a mediator of normal epithelial cell attachment and spreading. These studies extend the recent observations that normal keratinocytes synthesize and secrete TSP (11) and that in vivo, TSP is localized at the dermal-epidermal junction and in the basement membrane zone around sweat glands (14). These are sites where actively proliferating keratinocytes are in contact with the basement membrane. The data in the present study demonstrates that TSP induces the attachment and spreading of normal human keratinocytes and that undifferentiated, rapidly proliferating keratinocytes in culture are more adhesive than are keratinocytes that have been maintained under conditions which induce differentiation. The data indicate that the behavior of normal proliferating keratinocytes in response to TSP is similar to that of their malignant counterpart cells.

Methods

Cells. Normal human skin was obtained either from face-lift surgery or abdominoplasty, and prepared as single cell suspensions according to the method of Liu and Karasek (15). Small, round, viable cells (1.7–2.0 $\times$ 10^6) were seeded onto 35-mm culture dishes (Lux, Flow Laboratories, McLean, VA) as previously described (16). Growth was at 37°C and 5% CO_2. The culture medium consisted of MCDB 153 medium (Clonetics, San Diego, CA). This is a serum-free culture medium supplemented with 5 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.1 mM ethanolamine, and 0.1 mM phosphoethanolamine. MCDB 153 medium contains 0.3 mM Ca^{2+}. This medium has been shown to maintain normal keratinocytes in a rapidly growing and undifferentiated condition (17).

In addition to normal keratinocytes, a line of human squamous carcinoma cells entitled UM-SCC-11B was also used. The UM-SCC-11B line was established from laryngeal carcinoma tissue removed from a 65-yr-old male. This line was used in our previous studies with TSP and was identified as the most responsive among a group of

1. *Abbreviation used in this paper:* TSP, thrombospondin.

squamous carcinoma lines to TSP (10). In the present study, the squamous carcinoma cells were grown in monolayer culture at 37°C and 5% CO_2. The growth medium was Eagle's MEM with Earl's salts supplemented with nonessential amino acids, 15% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (MEM).

Reagents

TSP and its proteolytic fragments. TSP was purified from the releasate of thrombin-stimulated platelets by a combination of heparin-Sepharose and gel filtration chromatography as described previously (3). The 140-kd fragment was generated by subjecting TSP to digestion with 0.1% wt/wt chymotrypsin (60 U/mg, Sigma Chemical Co., St. Louis, MO) in 20 mM Tris, pH 7.6, 0.15 M NaCl, and 1 mM $CaCl_2$ for 20 min at 37°C. The digestion was stopped by the addition of PMSF to a final concentration of 1 mM. The resulting digest was fractionated by heparin-Sepharose chromatography (18). The nonheparin-binding 140-kd fragment was present in the column runthrough. The 25-kd heparin binding fragment was eluted with high salt–containing buffer (20 mM Tris, pH 7.6, 0.6 M NaCl).

The 120/18-kd fragment, which are in disulphide linkage, were formed by letting the chymotrypsin digest proceed longer (40 mins) using the same enzyme substrate ratio, temperature, and buffer as described above for the generation of the 140-kd fragment. The nonheparin-binding 120/18-kd fragment was similarly resolved from the 25-kd heparin-binding fragment by heparin-Sepharose chromatography. All digests, column fractions, and purified fragments were examined by SDS-PAGE and quantitated by the method of Lowry et al. (19).

Polyclonal and monoclonal antibodies to TSP. Rabbit polyclonal antibodies to TSP and three monoclonal antibodies to TSP were used in these studies. The monoclonal antibodies used were designated as antibody A2.5, directed against the heparin-binding domain of the TSP molecule, antibody C6.7, directed against the platelet-binding domain of the TSP molecule, and antibody A4.1, which binds to a 45-kd trypsin-resident core of the TSP molecule. The isolation and characterization of these antibodies have been described previously (3, 18, 20).

Laminin and fibronectin. Laminin was used in certain experiments. The laminin was prepared from the Englebreth-Holm-Swarm tumor by the method of Timpl et al. (21). Purity of the laminin was assessed by SDS-PAGE and by ELISA (22). When examined on a 7% SDS–polyacrylamide gel under reducing conditions, only two protein bands (at 200 and 400 kd) were seen. The laminin reacted by ELISA with monospecific polyclonal anti–laminin antibodies at dilutions up to 1:10^6 but did not react with antibodies to type IV collagen or fibronectin. Laminin prepared in this manner stimulated attachment and spreading of a laminin-responsive murine fibrosarcoma cell line at concentrations as low as 0.5 μg/ml (22).

Human plasma fibronectin was also used in certain experiments. It was obtained from Gibco (Grand Island, NY). When examined by SDS-PAGE under reducing conditions, a single major band (200 kd) was seen. The purified fibronectin reacted with monospecific polyclonal rabbit anti–fibronectin antibodies at dilutions up to 1:10^6 but did not react with anti–laminin antibodies. Fibronectin stimulated attachment and spreading of a fibronectin-responsive murine fibrosarcoma cell line at concentrations as low as 0.5 μg/ml.

Heparin. Heparin was obtained from Sigma Chemical Co.

Arg-Gly-Asp-Ser (RGDS). The tetrapeptide RGDS was obtained from Peninsula Laboratories, Inc. (Belmont, CA).

OKM5. Monoclonal antibody OKM5 was obtained from Ortho Diagnostic Systems, Inc. (Raritan, NJ). This antibody identifies an 88-kd cell surface antigen on platelets and monocytes that has recently been reported to be a TSP receptor (23).

Adhesion assay. Cell-substrate attachment and spreading were measured as described in our recent report (10). Briefly, various amounts of TSP, fragments of TSP, or control proteins were incubated for 2 h in wells of a 24-well cell culture dish under serum-free conditions. After this, the wells were washed and incubated with serum-free

MEM supplemented with 1% BSA. Cells to be used in the adhesion assay were harvested by trypsinization and added to the wells in MEM containing 1% BSA 15 min later. At various times thereafter, the nonattached cells were removed from the wells and counted with an electronic particle counter. The wells were then washed twice and fixed by the addition of 2% glutaraldehyde. The percentage of attached cells that were also spread was determined using a phase-contrast microscope with a calibrated grid in the eyepiece.

ELISA. ELISAs were performed to quantify the amount of immunoreactive TSP secreted into the culture medium by keratinocytes or squamous carcinoma cells maintained under various conditions. Briefly, 2-h culture fluids were harvested, clarified by low-speed centrifugation, and added to wells of a 96-well plate (Falcon Plastics, Cockeysville, MD) from lots that had been prescreened for acceptability in ELISAs. We used 200 μl per well and incubated the wells for 4 h at 37°C. Culture medium served as the control. Purified human platelet TSP (0.5–0.0005 μg/well) was also added to the assay plate to serve as a standard. After the 4-h incubation, the culture medium from the cells and the control culture medium were removed from the wells and the ELISA was performed as described previously (22). The cells from which the culture medium was obtained were harvested with trypsin and counted.

Results

Stimulation of keratinocyte attachment and spreading by TSP. Human keratinocytes were obtained from normal skin and established in culture in MCDB 153 medium. When cells were approaching confluency, they were divided into three groups. One group was incubated with fresh MCDB 153 medium (containing 0.3 mM Ca^{2+}). The second group was incubated in MCDB 153 medium supplemented with 1.4 mM Ca^{2+}, and the third group was incubated in MEM. Previous studies by others have shown that Ca^{2+} concentrations > 1.0 mM induce keratinocyte differentiation, as evidenced by the presence of cornified envelopes and stratification in conjunction with a reduction in growth (17). In our experiments, we observed a significant difference in morphology between the cells maintained under different conditions. The keratinocytes grown in MCDB 153 medium had the appearance of small, cuboidal, or elongated cells with a slight amount of cytoplasm. They were similar in appearance to the UM-SCC-11B cells. When the keratinocytes were grown for one day in MCDB 153 medium supplemented with 1.4 mM Ca^{2+} or in MEM, they were much more polygonal in shape, with more prominant cytoplasm, and with early stratification typical of differentiated keratinocytes. These morphological features can be seen in Fig. 1.

Fresh keratinocytes were examined for attachment and spreading on plastic culture dishes and dishes coated with 25 μg TSP, laminin, or fibronectin (Fig. 2). A high percentage of the cells rapidly attached on the TSP-coated dishes under serum-free conditions and some of the attached cells spread. In contrast, a much lower proportion of the cells attached to plastic culture dishes or dishes coated with either laminin or fibronectin. Furthermore, the kinetics of attachment were much slower on these substrates.

Keratinocytes established in culture were also examined for attachment and spreading on plastic culture dishes and TSP-coated culture dishes (Table I). Cells established in culture in MCDB 153 medium rapidly attached on TSP-coated dishes (i.e., within 1 h), and many of the attached cells also spread. The addition of 100 μg at anti-TSP to the buffer along with the cells inhibited adhesion while normal rabbit globulin had no effect. Keratinocytes established in culture also at-
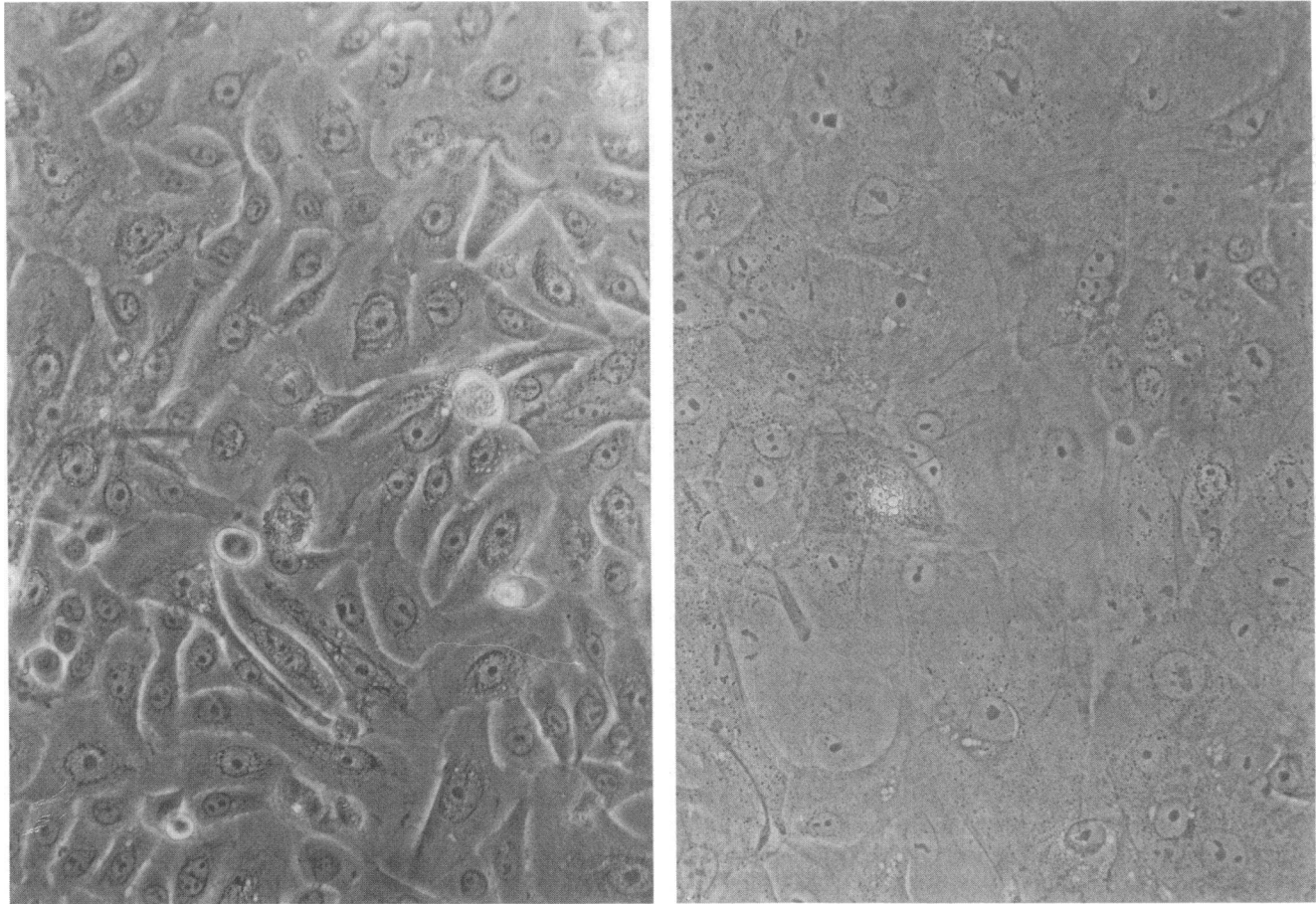
Figure 1. Morphology of keratinocytes grown under different culture conditions. Cells established in culture on MCDB 153 medium were incubated in the same medium (*left*) or were cultured for 1 d in MEM (*right*). The cells were then photographed under phase-contrast microscopy. × 320.
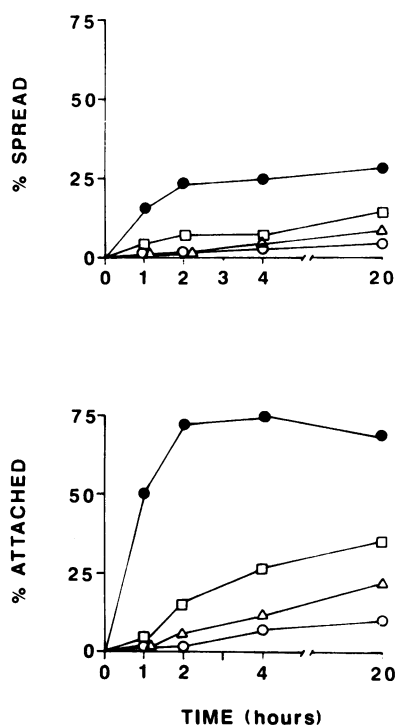
tached and spread on plastic culture dishes in the absence of exogenous adhesion factors. However, attachment and spreading were much slower on plastic culture dishes. Spreading, in particular, was slow on this substratum. As was observed on dishes coated with TSP, attachment and spreading on plastic culture dishes was partially inhibited by rabbit anti-TSP but not by normal rabbit globulin (Table I).

The relationship between keratinocyte differentiation and adhesion was examined next. When cells that had been initially established in MCDB 153 medium were cultured for 1 d in MCDB 153 supplemented with 1.4 mM Ca^{2+} or in MEM, and then examined for attachment and spreading on plastic culture dishes, they were less responsive than cells maintained in MCDB 153 medium. Quantitative differences are shown in Fig. 3. Photomicrographs are shown in Fig. 4.

Stimulation of attachment and spreading by proteolytic fragments of TSP. Proteolytic fragments of TSP were examined for promotion of cell attachment and spreading in order to define the cell-binding region of the molecule. To obtain the proteolytic fragments, we used chymotryptic digestion of intact TSP as described in the Methods. Mild chymotryptic digestion of TSP results in the formation of a 25-kd heparin-binding domain and a 140-kd nonheparin-binding fragment (18). The 140-kd fragment can be purified from the 25-kd heparin-binding fragment (Fig. 5, lane 2 *A*) by heparin-Sepha-

rose affinity chromatography (Fig. 5, lane 1 *B*) (3). Upon further exposure to chymotrypsin, the 140-kd fragment is proteolytically digested to give a 120- and an 18-kd fragment (Figure 5, lane 1, *A* and *B*) (18). The 120-kd fragment is in disulphide linkage with the 18-kd fragment (18), and the two together can be isolated in a purified form as described in Methods. The amino terminal sequence of the 140-kd fragment is identical to that of the 120-kd fragment (18). Thus, when the 140-kd species transits to the 120-kd species, the proteolytic cleavage that gives rise to the 18-kd fragment must occur at the carboxy terminus.

Using keratinocytes maintained on MCDB 153 medium and UM-SCC-11B squamous carcinoma cells, we examined the cells for attachment and spreading on dishes coated with 20 μg of the various proteolytic fragments of TSP. The 140-kd fragment of TSP stimulated attachment and spreading of both the normal and malignant epithelial cells. The kinetic data obtained with this fragment as well as the dose-response data were very similar to the data with the intact molecule and the percentages of cells responding to this fragment were the same as with the intact molecule (Table II). In contrast, when the 140-kd fragment was converted into a 120-kd fragment by release of an 18-kd fragment, attachment and spreading activity for both cell types was lost (Table II). Likewise, the isolated 25-kd heparin-binding fragment did not support attachment

Figure 2. Attachment and spreading of fresh keratinocytes on plastic culture dishes and dishes coated with 25 μg of TSP, laminin, or fibronectin. Attachment and spreading were measured as described in Methods. The attachment values are averages based on duplicate dishes in a single experiment. The individual values were within 15% of the average values. The spreading values are averages based on four fields in each of duplicate dishes in a single experiment. The standard deviations were within 10% of the average values. The experiment was repeated three times with similar results. Plastic (o); TSP (●); laminin (△); fibronectin (□).

carried out as described in the Methods, except that the dishes were incubated with the appropriate amount of each antibody for 1 h (and then washed) immediately before addition of the cells. As shown in Table IV, antibody A2.5 had no effect on attachment and spreading induced by either the intact molecule or the 140-kd fragment, while antibody A4.1 was highly effective against both ligands, and antibody C6.7 was intermediate.

In addition to the monoclonal antibodies directed against various parts of the TSP molecule, we also examined monoclonal antibody OKM5 for inhibition of TSP-induced attachment and spreading. This antibody recognizes an 88-kd antigen on the surface of human platelets and monocytes that has recently been identified as a TSP receptor (23). In our studies, 25 μg of the antibody was preincubated with the cells for 15 min and then added along with the cells to TSP-coated dishes. The percentage of attached and spread cells was determined 1 h later in the normal manner. Under these conditions, the antibody had no effect on attachment or spreading (Table IV). Note that we were not able to detect OKM5 binding to normal or malignant epithelial cells using immunofluorescence techniques. In contrast, binding of the same antibody to human monocytes was consistently observed (not shown).

The tetrapeptide RGDS, containing the cell recognition sequence from fibronectin (24), was also examined as a potential inhibitor of TSP-induced attachment and spreading. It has recently been shown that the RGD sequence occurs in TSP (25) as it does in several other adhesion factors (26). For these studies, 350 μg of the RGDS peptide was incubated for 15 min with the cells in a 1-ml vol. After this, the cells and the peptide were added to TSP-coated dishes and the percentage of attached and spread cells determined in the normal manner. Under these conditions, attachment and spreading were reduced only very slightly (Table IV). In contrast, when the cells (UM-SCC-11B cells) were examined on dishes coated with 25 μg of human plasma fibronectin, the same amount of RGDS in the buffer inhibited attachment and spreading by > 75% (not shown).

and spreading of either cell type. Although neither the 120/18-kd fragments or the 25-kd fragment induced attachment or spreading, they also failed to act as competitors. They did not alter the attachment and spreading of either cell type on plastic culture dishes in the presence of BSA. Likewise, they did not inhibit attachment or spreading on intact TSP (Table III).

Monoclonal antibodies that recognize different parts of the TSP molecule were examined for ability to inhibit attachment and spreading on dishes coated with intact TSP or the 140-kd fragment of TSP. In these experiments, the adhesion assay was

Table I. Modulation of Keratinocyte Attachment and Spreading

Treatment	Attached§ (1 h)	Spread‖ (1 h)	Attached (18 h)	Spread (18 h)
		%		
None*	0	—	82±4	76±5
100 μg normal rabbit globulin	0	—	75±6	82±1
100 μg anti-TSP	0	—	28±4	11±4
25 μg TSP‡	55±3	30±2	ND	
25 μg TSP + 100 μg normal rabbit globulin	52±8	22±5	ND	
25 μg TSP + 100 μg anti-TSP	10±1	0	ND	

* Attachment and spreading on plastic culture dishes in the presence of BSA. In this experiment, 100 μg of rabbit globulin or 100 μg of anti-TSP was added to the reaction buffer containing the harvested and washed cells in suspension and incubated for 15 min. After this, the entire contents were transferred to the wells and the percentages of cells that were attached and spread determined after 1 and 18 h. ‡ In this experiment, wells of a 24-well culture dish were treated with 25 μg of TSP for 2 h at 37°C. After this, the nonabsorbed TSP was removed and the wells washed. The cells were added and the assay carried out in the normal way. In the wells treated with antibodies, the wells were first treated with 25 μg of TSP and incubated for 2 h at 37°C. After removal of the nonabsorbed TSP and washing, the wells were treated with 100 μg of either normal rabbit globulin or anti-TSP and incubated for an additional 1 h at 37°C. The wells were then washed again and the cells added.
§ Attachment values are averages plus or minus the differences between individual values and averages in a single experiment. The experiment was repeated two times with similar results. ‖ Values for spreading are average±SD based on four areas in each of duplicate dishes in a single experiment. The experiment was repeated two times with similar results.

TIME (hours)
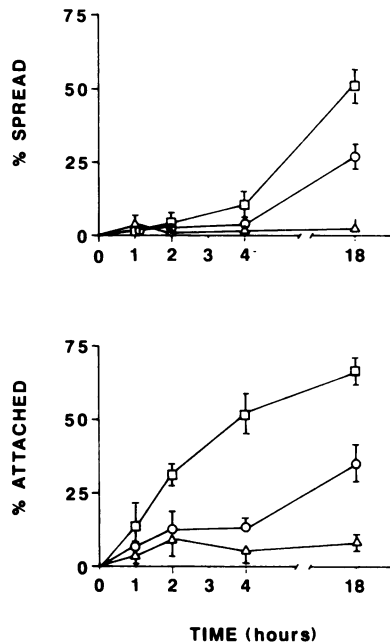
Figure 3. Modulation of keratinocyte attachment and spreading on plastic culture dishes. Keratinocytes were established in culture in MCDB 153 medium and divided into three groups. One group was incubated for 1 d in the same medium. The second group was incubated for 1 d in MCDB 153 medium supplemented with 1.4 mM Ca^{2+}, and the third group was incubated for 1 d in MEM. After the incubation, the cells were harvested and examined for attachment and spreading on plastic dishes in the presence of BSA. Attachment values are averages plus or minus the differences between the individual values and the averages based on two dishes. Values for spreading are averages±SD based on four fields in each of duplicate dishes. The experiment was repeated three times with similar results. MCDB 153 (□); MCDB 153 + 1.4 mM Ca^{2+} (○); MEM (△).

Heparin was also examined for inhibition of attachment and spreading. When this agent was used, it was added at 100 μg per reaction to the TSP-coated wells or wells coated with the 140-kd fragment along with the cells. This treatment did not alter attachment or spreading of either the keratinocytes or squamous carcinoma cells on either substrate (Table IV).

Production of TSP by keratinocytes and squamous carcinoma cells. Normal human keratinocytes and human squamous carcinoma cells have been shown in previous studies to biosynthesize TSP (10, 11). The synthesized TSP is secreted into the culture medium and bound to the cell surface (10, 11). In the present study we used a sensitive ELISA to quantitate the amount of TSP secreted into the culture medium by keratinocytes maintained under various conditions and by squamous carcinoma cells. Keratinocytes were established in culture on MCDB 153 medium. When cultures were approaching confluency, they were washed twice and incubated for an additional 20 h in MCDB 153 medium (0.3 mM Ca^{2+}), in MCDB 153 medium supplemented with 1.4 mM Ca^{2+}, or in MEM (1.4 mM Ca^{2+}). After the 20-h incubation, the cultures were again washed and incubated for 2 h in MCDB medium. The 2-h culture fluids were then examined for immunoreactive TSP by ELISA. Culture fluids from squamous carcinoma cells maintained in culture on MEM were examined in the same manner. Comparable amounts of immunoreactive TSP were found in the culture fluid of keratinocytes maintained in MCDB 153 medium and squamous carcinoma cells, while much less was found in the culture fluid of keratinocytes maintained in MCDB 153 medium in the presence of 1.4 mM Ca^{2+} or in MEM (Table V). In contrast to these results with keratinocytes in culture, freshly isolated keratinocytes produced barely detectable amounts of immunoreactive TSP when incubated for 2 h in MCDB 153 medium at concentrations as high as 2×10^6 cells per 1.5 ml of culture medium (Table V).

Discussion

This study was undertaken to examine the role of TSP in cell-substrate adhesion of normal keratinocytes. Exogenous TSP induced attachment and spreading of freshly isolated keratinocytes and keratinocytes maintained in culture. TSP was
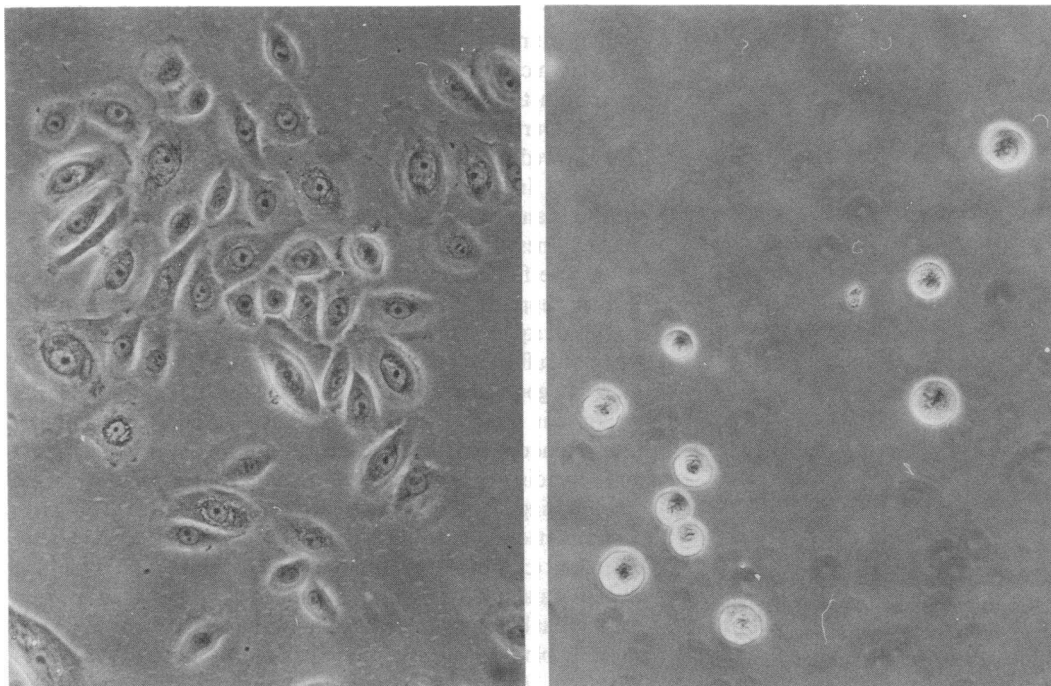
Figure 4. Morphology of keratinocytes on plastic culture dishes 18 h after plating. Cells grown for one day in MCDB 153 medium (*left*) or cells grown for one day in MEM (*right*) were harvested from culture and examined for attachment and spreading as described in Methods. After 18 h, the nonattached cells were removed and the remaining cells photographed under phase-contrast microscopy. × 280.
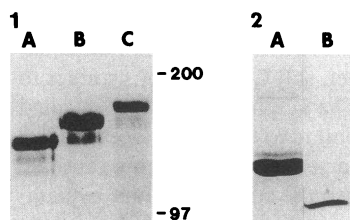
Figure 5. SDS-PAGE of TSP and fragments. Intact TSP (lane 1 *C*) and its two chymotryptic fragments, 140 kd (lane 1 *B*) and 120 kd (lane 1 *A*) are shown resolved on a 5% SDS-acrylamide gel. Purified 25 kd heparin binding domain (lane 2 *A*) and 18-kd fragment (lane 2 *B*) are shown resolved on a higher percentage (15%) SDS acrylamide gel. The 120- and 18-kd fragments are in disulphide linkage, but only the 120-kd fragment is visualized on the 5% acrylamide gel and the 18-kd fragment on the 15% gel due to their disparate molecular weights. All samples were reduced before loading with 5% vol/vol 2-mercaptoethanol and gels were stained with Coomassie Blue. Molecular mass standard positions are indicated by the numbers that are in kilodaltons.

more effective than either laminin or fibronectin. In this regard, keratinocytes are similar to their malignant counterpart (squamous carcinoma) cells (10, 13). They are also similar to squamous carcinoma cells in that they synthesize TSP endogenously (10, 11) and secrete immunoreactive TSP into the culture medium (references 10 and 11, and this report). Our data suggest that the endogenously synthesized TSP may play an

Table II. Stimulation of Attachment and Spreading by Intact TSP and by Proteolytic Fragments of TSP

Ligand	Squamous carcinoma		Keratinocytes	
	Attached	Spread	Attached	Spread
	%			
None	16±2	0	5±2	0
Intact TSP				
20 μg	93±5	87±3	87±3	37±5
10 μg	95±4	85±7	82±5	27±3
5 μg	95±6	90±1	85±4	30±9
1 μg	80±2	61±5	54±6	19±2
0.5 μg	45±6	17±10	21±8	10±5
140-kd fragment				
20 μg	90±6	80±6	90±11	40±5
10 μg	94±13	84±2	85±4	31±3
5 μg	99±7	72±10	72±10	30±8
1 μg	30±7	16±2	27±5	15±4
0.5 μg	27±11	5±2	10±1	5±2
120-kd fragment				
100 μg	12±6	7±5	10±3	0
50 μg	22±5	5±1	9±3	0
25-kd fragment				
100 μg	22±5	3±1	10±5	0
50 μg	15±7	6±3	12±4	0

Attachment and spreading were measured as described in Methods. The percentage of attached cells was determined from duplicate dishes 1 h after plating. The values shown are averages plus or minus the differences between the individual values and the averages. The percentage of spread cells was determined based on four readings in each of duplicate dishes. The values shown are averages±SD. The experiment was repeated three times with similar results.

Table III. Failure of the 120/18- and 25-kd Fragments to Inhibit Attachment and Spreading Induced by TSP

Ligand	Attached	Spread
	%	
TSP (1 μg)	85±2	37±3
TSP (20 μg)	88±2	80±4
TSP (1 μg)		
+50 μg 120/18 kd	80±6	30±3
+50 μg 25 kd	91±2	25±8
TSP (20 μg)		
+50 μg 120/18 kd	90±7	82±6
+50 μg 25 kd	83±9	79±5

Wells of a 24-well plate were coated with intact TSP for 2 h. At the time of assay, 50 μg of the 120/18-kd fragment or 50 μg of the 25-kd fragment were added along with the cells. Attachment and spreading of squamous carcinoma cells were measured as described in the Methods. The percentage of attached cells was determined from duplicate dishes 1 h after plating. The values shown are averages plus or minus the differences between the individual values and the averages. The percentage of spread cells was determined based on four readings in each of duplicate dishes. The values shown are averages±SD. The experiment was repeated three times with similar results.

important role in cell-substrate adhesion of normal keratinocytes as well as squamous carcinoma cells. Among keratinocytes, there was a direct relationship between TSP production and attachment and spreading on plastic culture dishes. Freshly isolated keratinocytes produced minimal amounts of TSP and < 10% of these cells attached and spread on plastic dishes in the presence of BSA. When the cells were established in culture and maintained in MCDB 153 medium they produced much higher amounts of TSP. A high percentage of these cells (~ 70%) attached on plastic culture dishes in the absence of serum or other source of exogenous adhesion factors. When the cells in culture were grown for 1 d in MCDB 153 medium supplemented with high Ca^{2+} (1.4 mM) or in MEM, which also contains 1.4 mM Ca^{2+}, TSP production fell in conjunction with the capacity of the cells to attach and spread. Our previous studies with squamous carcinoma cells also showed a direct relationship between TSP synthesis and adhesion. In that study, a direct relationship between TSP synthesis and cell-substrate adhesion was found among different cell lines (10).

A role for endogenous TSP in cell-substrate adhesion is further supported by the finding that both normal keratinocytes and squamous carcinoma cells produce amounts of TSP that are sufficient to induce cell-substrate adhesion, and that there is a good correlation between the kinetics of the adhesion response in the absence of exogenous TSP and the accumulation of endogenous TSP in the culture medium. Attachment and spreading of both cell types first become evident by 2–4 h after plating. By this time sufficient amounts of TSP have accumulated in the culture medium to induce attachment and spreading (compare the dose-response data in Table II and the ELISA data in Table V). Additionally, it has already been shown that basal adhesion of squamous carcinoma cells can be partially blocked with polyclonal or monoclonal antibodies to TSP (10). Note that squamous carcinoma cells are not unique

Table IV. Inhibition of Attachment and Spreading Induced
by Intact TSP and by the 140-kd Fragment of TSP*

Condition	Squamous carcinoma		Keratinocytes	
	Attached	Spread	Attached	Spread
	%			
None	10±2	0	10±5	0
25 μg TSP	80±2	65±2	68±8	29±3
25 μg TSP +				
100 μg heparin	88±3	73±3	75±12	33±3
100 μg Mab A2.5	87±5	64±2	ND	ND
25 μg Mab A2.5	96±6	60±3		
100 μg Mab A4.1	25±3	18±3	35±3	10±3
25 μg Mab A4.1	20±1	10±2		
100 μg Mab C6.7	48±8	36±4	ND	ND
25 μg Mab C6.7	54±2	42±5		
25 μg OKM5	90±7	70±6	65±2	35±4
350 μg RGDS	68±4	52±6	68±7	30±3
25 μg of 140-kd fragment	75±5	68±6	60±7	34±4
25 μg of 140-kd fragment +				
100 μg heparin	80±3	70±3	ND	ND
100 μg Mab A2.5	79±4	70±3	ND	ND
25 μg Mab A2.5	70±5	70±5	ND	ND
100 μg Mab A4.1	20±4	24±4	ND	ND
25 μg Mab A4.1	16±7	25±1	ND	ND
100 μg Mab C6.7	56±8	42±6	ND	ND
25 μg Mab C6.7	48±7	45±7	ND	ND

Mab, Monoclonal antibody.
* Attachment and spreading were measured as described in Methods.
The percentage of attached cells was determined from duplicate
dishes 1 h after plating. The values shown are averages plus or minus
the differences between the individual values and the averages. The
percentage of spread cells was determined based on four readings in
each of duplicate dishes. The values shown are averages±SD. The ex-
periment was repeated three times with similar results.

in their utilization of endogenously produced adhesion factors.
Previous studies have shown that endogenously synthesized
laminin promotes attachment and spreading of murine tumor
cells (22, 27–29).

Efforts were begun in this study to identify the region of the
TSP molecule responsible for stimulating attachment and
spreading. It appears that attachment and spreading activity
for both the normal and malignant epithelial cells resides in a
140-kd fragment that is lacking the NH₂-terminal 25-kd hepa-
rin-binding domain. This was suggested by the lack of inhibi-
tion observed with a monoclonal antibody directed against the
heparin-binding domain (A2.5) or with heparin itself, and
confirmed directly by adhesion studies with purified frag-
ments. With both normal keratinocytes and squamous carci-
noma cells, the 140-kd fragment supported cell attachment
and spreading in a virtually identical manner to the intact TSP
molecule. In contrast, the 25-kd (heparin-binding) fragment
removed during the generation of the 140-kd moiety had no
effect on either attachment or spreading. This is different from
what Roberts, Sherwood, and Ginsburg (8) reported with
human melanoma cells. They showed that the 140-kd frag-
ment supported melanoma cell attachment but not spreading.

Table V. Immunoreactive TSP in the Culture Fluid
of Keratinocytes and Squamous Carcinoma Cells

Cells	TSP (μg/ml/10⁶ cells)‖
Fresh keratinocytes*	<0.10
Cultured keratinocytes‡	
MCDB 153	0.51±0.03
MCDB 153 (1.4 mM Ca²⁺)	0.19±0.09
MEM	0.11±0.05
Squamous carcinoma cells§ (MEM)	0.56±0.11

* Fresh keratinocytes (2 × 10⁶) were incubated for 2 h in 1.5 ml of
MCDB 153 medium. The medium was harvested and examined for
immunoreactive TSP by ELISA.
‡ Keratinocytes (3 × 10⁵) established in culture in MCDB 153 me-
dium were incubated for 1 d in MCDB 153 medium, MCDB 153
medium supplemented with 1.4 mM Ca²⁺, or MEM. After this, the
dishes were washed and incubated for 2 h in MCDB 153 medium.
The 2-h culture fluids were harvested and examined for immunore-
active TSP by ELISA. The cells from the dishes were harvested and
counted.
§ Squamous carcinoma cells (3 × 10⁵) were maintained in culture in
MEM. They were washed and incubated for 2 h in MCDB 153 me-
dium. The 2-h culture fluid was harvested and examined for immu-
noreactive TSP by ELISA. The cells were harvested and counted.
‖ The values shown are averages plus or minus the differences be-
tween the individual values and the averages based on duplicate
dishes in a single experiment. The experiment was repeated three
times with similar results.

Spreading activity was presumed to reside in the 25-kd hepa-
rin-binding domain because a monoclonal antibody directed
against the heparin binding domain of the TSP molecule (the
same monoclonal antibody used in the present study) inhib-
ited spreading but not attachment. While these differences
may indicate that epithelial cells and melanoma cells use dif-
ferent parts of the TSP molecule to spread, an alternate expla-
nation is that the epithelial cells do not need the exogenous
25-kd fragment for spreading because they are able to synthe-
size enough of it themselves. It has been shown in previous
studies (10, 11) as well as in the present study that normal
keratinocytes and squamous carcinoma cells are very active in
terms of TSP production. In contrast, we were unable to detect
TSP biosynthesis by melanoma cells in our recent study (10).

Where the cell-binding domain resides within the 140-kd
fragment of TSP is not known. It is known, however, that
when the 140-kd fragment transits to a 120- and 18-kd frag-
ment, ability to support attachment and spreading is lost. This
suggests that neither the 120-kd fragment or the 18-kd frag-
ment contains the cell recognition sequence and that the short
segment of amino acids that is lost in the conversion of the
140-kd fragment to the 120- and 18-kd fragment may be criti-
cal. This possibility is presently under investigation.

The findings described here may be directly relevant to
keratinocyte behavior in vivo. The low level of TSP produced
by the freshly isolated epidermal cells (a high proportion of
which are already differentiated) and by the cells that were
induced to differentiate may explain the reduced adhesiveness
of these cells in vivo. In vivo, keratinocytes located in the basal
cell layer are responsible for proliferation. When these cells
differentiate, they lose their attachment to the basement

membrane, migrate from the basal cell layer, and occupy suprabasalar locations. The findings described here may also be relevant to the complex events that occur during cutaneous wound healing. The increased synthesis of TSP by proliferating keratinocytes may provide (along with platelets) the matrix that is necessary for subsequent epithelialization. Perhaps TSP should be added to the list of important molecules, such as fibronectin and collagen, which mediate cell-matrix interactions in cutaneous wound healing (30–33).

Acknowledgments

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