A Cell Binding Domain from the α3 Chain of Type IV Collagen Inhibits Proliferation of Melanoma Cells*

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Our previous studies have shown that a peptide corresponding to the residue sequence 185–203 of the NC1 domain of the α3 chain of basement membrane collagen (type IV) inhibits the activation of polymorphonuclear leukocytes. Peptides from the same region of the α1, α2, α4, and α5(IV) chains did not exhibit this property. Because of the intimate relationship between metastasizing neoplastic cells and vascular as well as epithelial basement membranes, we measured the cell adhesion-promoting activity of peptides from the NC1 domain of type IV collagen and their effect on proliferation of human melanoma cells. We found that peptide α3(IV)185–203 (CNYYSN5SYFWSLASLNP88R) not only promotes adhesion of human melanoma cells but also inhibits their proliferation. Adhesion increased by 50–60% over control. Melanoma cell proliferation was inhibited by 40% when cells were grown in a medium containing 5 μg/ml peptide for 5 days. Studies showed that replacement of serine in position 189 or 191 by alanine resulted in significantly reduced adhesion. Similarly, serine replacement resulted in reduced ability to inhibit proliferation. Our data suggest that a region of the NC1 domain of the α3(IV) chain, contained within the sequence 185–203, not only specifically promotes adhesion but also inhibits proliferation of melanoma cells. These properties appear to be dependent on the presence of the triplet sequence -SNS- (residues 189–191), which is unique to the α3 chain and may represent an important functional epitope.

Type IV collagen is a major component of basement membranes. The predominant molecular species is a heterotrimer composed of two α1 and one α2 chain. The presence of additional type IV collagen chains, α3(IV), α4(IV), α5(IV), and α6(IV), has been reported (1–6). There is evidence that the latter are distributed in most basement membranes (4, 5). Type IV collagen not only forms the main structural framework of all basement membranes, but also serves as scaffolding for the binding of other basement membrane components (7, 8). One important function of type IV collagen is its ability to promote the adhesion and motility of various normal and transformed cell types (9).

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The α chain of type IV collagen has a long collagenous domain of about 1,400 amino acid residues and a non-collagenous domain of about 230 residues at the carboxyl terminus, called the NC1 domain. The NC1 domain is thought to play a key role in the alignment and selection of three α chains forming a triple helical molecule (10, 11). Several studies have focused on the biological activity of the NC1 domain of type IV collagen. One synthetic heparin-binding peptide, Hep-I, originating from NC1 domain of the α1 chain of type IV collagen and containing 12 amino acids, has been reported to promote the adhesion and spreading of bovine aortic endothelial cells (12, 13). A series of synthetic peptides from the NC1 domain of several α chains has been used to map antigenic epitopes on type IV collagen (14). One of these peptides, comprising residues 185–203 of the NC1 domain of the α3(IV) chain, has been shown to inhibit the activation of PMN1 as measured by a reduction in O2 production and proteolytic enzyme release (15).

Neoplastic cells have the ability to invade and metastasize. Because of the intimate relationship between epithelial cells and basement membranes as well as metastatic cells and vascular basement membranes we decided to examine the ability of the NC1 domain of type IV collagen and synthetic peptides from this domain to influence adhesion and proliferation of melanoma cells. Our studies show that a synthetic peptide comprising residues 185–203 of the NC1 domain of the α3(IV) chain promotes adhesion and inhibits proliferation of melanoma cells. Using monoclonal antibodies that recognize the above peptide, we have shown that there is a multifunctional domain within the first 12 amino acids of the residue sequence 185–203 capable of promoting the cell adhesion and inhibition of proliferation. Because synthetic peptides of the same region from the other α(IV) chains that lack the triplet -SNS- (residues 189–191) fail to inhibit melanoma cell adhesion and proliferation and because the sequence -SNS- is unique to the α3(IV) chain we can assume that this triplet represents the functional epitope of this peptide.

MATERIALS AND METHODS

Cell Culture—The human melanoma cell lines used in these studies, WM9, WM164, WM136 1A were kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia). Metastatic cell lines WM9 and WM164 were derived from an intermediate stage metastatic lesion. WM136 1A was obtained from a late lesion with concomitant distant metastases at the time of tumor removal (16, 17). The above cell cultures were grown in tumor medium (MCDE 153L-15, 4:1, Sigma) supplemented with 2% fetal bovine serum and insulin (5 μg/ml, Sigma). In addition, other tumor cell lines including two melanoma cell lines (HT-144 and UACC-903), a fibrosarcoma cell line (HT-1080), and an

1 The abbreviations used are: PMN, polymorphonuclear leukocytes; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
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Cell Proliferation Assay—The determination of cell proliferation was done by counting cells in a coulter counter or by determining the \[^{3}H\]thymidine incorporation as a measure of DNA synthesis, or by the MTT method. By using the MTT method, the activity of living cells via mitochondrial dehydrogenase activity was measured. MTT cell growth determination kit, Sigma. For competition of the \[^{3}H\]thymidine incorporation measurements, confluent cultures were dissociated and suspended in fresh tumor medium. 1 × 10^5 cells were added to wells of a 12-well plate and incubated for 3–4 h. Then, fresh medium containing one of the peptides was added to the wells. The medium was changed every 2 days. After a 5-day incubation with peptides, cells were either dissociated with 0.25% trypsin, EDTA and cell numbers counted in a coulter counter, or were labeled with \[^{3}H\]thymidine (3 μCi/ml) overnight. Labeled cells were lysed with 1% Triton X-100 in PBS and radioactivity measured in a scintillation counter.

To test whether it is the peptide binding to the cells in the medium which induces the inhibitory effect on cell proliferation or whether it is the peptide adsorbed to the well surface causing strong cell adhesion which inhibits cell proliferation, cell-peptide interaction experiments were carried out. In one experiment, peptides were coated at varying amounts onto the wells of a 96-well plate, and 5 × 10^3 cells were added to each well. After a 5-day incubation, the MTT solution in an amount equal to 10% of the culture volume was added to each well, according to the kit procedure (Sigma). After a 4-h incubation at 37 °C, the culture medium was removed, and 0.5 ml of dissolution solvent in an amount equal to the original culture volume was added to each well. The plates were read at 570 nm in a Microplate reader EL340 (Bio-Tek Instruments), and cell proliferation was determined. The cells in uncoated wells incubated with the medium containing the peptide were used as control. In another experiment, we tested whether the peptide from the medium adsorbed onto the well surface. Cells were removed by 0.1% sodium dodecyl sulfate after a 5-day incubation with the medium containing varying amounts of the \[^{3}H\]thymidine-185–203 peptide. The presence of peptide attached to the well was detected by adding to the wells a polyclonal antibody to the \[^{3}H\]thymidine peptide at 1:250 dilution and incubating for 1 h at room temperature. The immunoreactivity of the \[^{3}H\]thymidine peptide was detected by ELISA as described above.

RESULTS

Characterizations of Monoclonal Antibodies—To characterize the monoclonal antibodies that recognize a functionally important domain within the peptides of the NC1 domain of the \[^{3}H\]thymidine chain of type IV collagen, we determined the ability of monoclonal antibodies to bind selectively to the synthetic peptides from the partial sequences of the \[^{3}H\]thymidine chain and from the comparable region of the different α chains. As shown in Table I, the polyclonal antibody and monoclonal antibodies A5B7 and A5D7 reacted strongly only with the synthetic peptides from the \[^{3}H\]thymidine chain, i.e. \[^{3}H\]thymidine 185–208 and \[^{3}H\]thymidine 185–203, but did not react with the peptides from comparable regions of α1(IV), α2(IV), and α5(IV) chains, even though they share considerable homology. To determine the critical sequence recognized by the monoclonal antibodies, the partial sequences of the \[^{3}H\]thymidine peptide, i.e. peptides comprising residues 185–196 and 194–203, were tested. Both the polyclonal antibody and the monoclonal antibodies A5B7 and A5D7 reacted strongly with the peptide \[^{3}H\]thymidine 185–196, but they reacted very weakly with the peptide \[^{3}H\]thymidine 194–203. The data indicate that the epitope recognized by the antibodies is contained within the region of residues 185–196 of the \[^{3}H\]thymidine chain. It should be noted that the major difference between the \[^{3}H\]thymidine 185–196 peptide and comparable peptides from other α chains in this region is the sequence -SNS- (residues 189–191) within the \[^{3}H\]thymidine peptide which is not present in the others (Table I). It appears that this unique -SNS- sequence within the \[^{3}H\]thymidine peptide is very critical. Peptides where one or both serines are substituted (α1, α2, or α5) failed to react with the antibodies (Table I).

It is interesting to note that the monoclonal antibody D12H5 reacted very weakly with all of the peptides, and it was therefore used as the control antibody in the competition of cell binding assay.
Effect of Peptides on Cell Proliferation—Table II shows the effects of synthetic peptides on tumor cell proliferation. Proliferation of melanoma cells was inhibited from 20 to 42% when peptide a3(IV)194–203 did not mediate cell adhesion beyond that of control. The property of cell adhesion is, therefore, dependent on the sequence of the NC1 domain of the a3(IV)185–203 peptide.

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**TABLE I**

Amino acid sequence of synthetic peptides from the NC1 domain of type IV collagen and immunologic reactivity by ELISA of the peptides with monoclonal and polyclonal antibodies (A405 nm).

| Peptides | Amino acid sequences | Antibodies |
|----------|----------------------|------------|
|          |                      | Monoclonal A5B7 | Monoclonal A5D7 | Monoclonal D12H5 | Polyclonal |
| a3(IV) 179–208 | CHGRTGCNYYSNSYSFWLASLNPEREFP | 0.97 | 1.61 | 0.20 | >2 |
| a3(IV) 185–203 | CNYYNSYSFWLASLNPER | 1.54 | 1.61 | 0.15 | >2 |
| a1(IV) 185–203 | CNYYANAYSFWL | 0.11 | 0.11 | 0.11 | 0.11 |
| a2(IV) 185–203 | CHYANKYSFWLTIPEQS | 0.09 | 0.08 | 0.09 | 0.08 |
| a5(IV) 182–200 | CNYYNSYSFWL | 0.09 | 0.10 | 0.09 | 0.09 |
| a3(IV) 185–196 | CNYYNSYSFWL | 0.64 | 1.0 | 0.11 | 1.20 |
| a3(IV) 194–203 | FWLASLNPER | 0.21 | 0.24 | 0.09 | 0.34 |
| a3(IV) L5 clone 1–15 | GQLDLAFVNLRS | 1 | 1 | 5 |
| a4(IV) 182–200 | CHFANKSFNITTVPLQ | 182 | 200 |
| a3(IV) 185–203 | CVYRNSYSFWLASLNPER | 185 | 203 |
| 189 S → A | CVYRNSYSFWLASLNPER | 194 | 203 |
| 191 S → A | CVYRNSYSFWLASLNPER | 185 | 196 |

* Immunologic reactivities of these peptides were not tested by the ELISA.

**Fig. 1.** Attachment of human melanoma cells, WM9 and WM136 1A. Each well of 24-well plates was coated with synthetic peptide a1(IV)185–203, a2(IV)185–203, a3(IV)185–203, a5(IV)182–200, and partial sequences of a3(IV)185–203, a3(IV)185–196, and a3(IV)194–203 (2.5 μg/well) overnight. 2.5 × 10⁵ [³⁵S]methionine-labeled cells were added to each well, and attached cells were measured as described under “Materials and Methods.” ■, control; □, a1(IV)185–203; △, a2(IV)185–203; ■, a3(IV)185–196, a3(IV)194–203. Error bars represent S.D. from the mean.
containing medium was removed, and cells were incubated in peptide-free medium for an additional 6 days. At the end of this period, cell proliferation was back to control levels (Fig. 2d).

Prevention of Cell Adhesion and Inhibition of Proliferation by Antibodies—To test the specificity of the cell binding domain of the peptide α3(IV)185–203, both monoclonal and polyclonal antibodies against this sequence were used to block peptide-mediated cell adhesion and inhibition of cell proliferation. WM9 melanoma cell adhesion on surfaces coated with peptide α3(IV)185–203 (2.5 μg/well) was monitored in the presence of anti-peptide antibodies. Melanoma cell adhesion was inhibited to an extent of 53–60% by the polyclonal antibody and by the monoclonal antibody A5D7. No significant inhibition of cell adhesion was observed in the presence of a control nonreactive monoclonal antibody D12H5 (Fig. 3a). The effect of the α3(IV) peptide on cell proliferation, on the other hand, was also prevented by the antibodies. When the α3(IV) peptide was treated with antibody before adding to the medium, its inhibitory effect on cell proliferation was decreased significantly (Fig. 3b). It is suggested that a functional domain in the α3(IV)185–203 peptide, recognized by the monoclonal antibody A5D7, is responsible for the activities of promotion of cell adhesion and inhibition of cell proliferation.

Identification of the Functional Domain in α3(IV) Peptide—
The current studies indicate that the sequence of residues 185–196 of the NC1 domain of the α3(IV) chain contains the functional domain that is responsible for the cell adhesion and the inhibitory effect on cell proliferation. The unique triplet -SNS- within the above sequence appears to represent such a functional domain. To confirm this, we replaced serine with -SNS- within the above sequence and tested the effect on melanoma cell proliferation. The unique triplet sequence that significantly increases adhesion of melanoma cells is the triplet -SNS-. Replacement of serine with alanine in either position 189 or 191 abolishes both activities of the peptide.

### DISCUSSION

Our previous studies have shown that the peptide α3(IV)185–203 prevents activation of human PMN, and the unique sequence -SNS- is the domain required for the inhibition of O2 production and enzyme release by these cells (15). Because melanoma cells are in direct contact with basement membranes during tumor cell invasion and metastasis, we elected to assess the in vitro influence of type IV collagen peptides on the attachment and the proliferation of human melanoma cells. In this study, several synthetic peptides from the NC1 domain of the α chains of type IV collagen were tested. Residues 185–203 of the α3(IV) chain comprise the only sequence that significantly increases adhesion of melanoma cells and inhibits their proliferation. The peptide-mediated melanoma cell adhesion can be prevented by specific polyclonal and monoclonal antibodies. It is of interest to note that the monoclonal antibody that recognized the sequence 185–196 of the α3(IV) peptide also prevented the inhibition of melanoma cell proliferation. It would appear that the cell adhesion-promoting activity and the inhibitory effect of the peptide on cell proliferation may be two functions that are intimately related. To rule out the possibility that strong attachment of melanoma cells to the peptide in the substrata was responsible for the effect of the α3(IV) peptide on melanoma cell proliferation, we carried out cell-peptide interaction experiments. The data clearly showed that it was the binding of the peptide to the apical region of the cells rather than to the basal region which was responsible for inhibition of cell proliferation (Fig. 2, b and c). We also observed that exposure of the cells to the peptide caused aggregation and clumping (data not shown). It would appear that it is the interaction of the cell surface with the soluble peptide which is necessary for inhibition of cell proliferation to occur.

In the current studies, it appears that the sequence -SNS- of the α3(IV)185–203 peptide is an absolute requirement for both cell adhesion and inhibition of cell proliferation. Although this region is highly conserved in the NC1 domain of all α chains of type IV collagen, the sequence -SNS- (residues 189–191) is unique to the α3(IV) chain. Both monoclonal and polyclonal antibodies specifically recognized the region of the peptide α3(IV)185–196 which contains the sequence -SNS-. The antibodies efficiently inhibited the cell binding to the substrate, confirming the specificity of this interaction. Substitution of either serine in the sequence -SNS- abolished the cell adhesion activity of the peptide, suggesting that the sequence -SNS- is a

### Table II

| Peptide       | α2(185–203) | α3(185–203) | α3(185–203) | α4(182–200) |
|---------------|-------------|-------------|-------------|-------------|
| α3(IV)185–203 | 100 ± 14    | 87.9 ± 8    | 87.2 ± 11   | 71.9 ± 3    |
| α3(IV)185–203 | 100 ± 7     | 102.3 ± 5   | 114.2 ± 10  | 80.0 ± 12   |
| α3(IV)185–203 | 100 ± 10    | 106.7 ± 5.3 | 94.5 ± 11.5 | 62 ± 8.6    |
| α3(IV)185–203 | 100 ± 4.5   | ND          | ND          | 68.7 ± 4    |
| α3(IV)185–203 | 100 ± 7.6   | ND          | ND          | 57.5 ± 6    |

**Cells (2.5 × 10^4/cm² well)** were incubated with various peptides and labeled with [3H]thymidine as described under "Materials and Methods."
multifunctional cell binding domain that mediates cell adhesion and inhibits cell proliferation.

Unpublished studies in our laboratory demonstrate that this peptide inhibits proliferation not only of melanoma cells but of other epithelial tumors cell and concomitantly increases intracellular cAMP. It was strongly suggested that a mechanism involving a signal transduction pathway may play a role in the cell adhesion-promoting activity and in inhibition of cell proliferation by the \( \alpha_3(IV) \) peptide. The same peptide causes an inhibition of PMN activation through a rise in intracellular cAMP (15). An increase in cAMP is associated with an inhibition of \( O_2^{2-} \) production as well as secretion of proteinases and lactoferrin (15, 22). The increase of cAMP in PMN (15) and in melanoma cells induced by the peptide can be inhibited by pertussis toxin, suggesting involvement of G proteins (23). A detailed study of the events in the signal transduction pathway triggered by the \( \alpha_3(IV) \) peptide is the subject of a manuscript under consideration. The direct effect of the \( \alpha_3(IV) \) peptide on melanoma cell adhesion and proliferation raises the question of whether a specific receptor for this peptide is involved. Studies are under way to isolate and characterize the putative receptor.

Tumor cell proliferation and metastasis are complex processes involving numerous tumor cell-extracellular matrix interactions that at present are incompletely understood. The interaction of tumor cells with basement membrane is the first step in a multifunctional process (24).

**FIG. 2. Effects of peptides on melanoma cell proliferation.** Panel a, WM9 cells were treated with various partial sequences of synthetic peptide \( \alpha_3(IV)185–203 \), i.e. \( \alpha_3(IV)185–196 \) and \( \alpha_3(IV)194–203 \) (5 \( \mu \)g/ml). ■, control; □, \( \alpha_3(IV)185–203 \); △, \( \alpha_3(IV)185–196 \); ○, \( \alpha_3(IV)194–203 \). Cell numbers were counted as described under “Materials and Methods” and expressed as percent of control. Panel b, to test the effect of cell attachment to substrate containing the peptide, WM9 cells were plated onto wells coated with various amounts of the \( \alpha_3(IV)185–203 \) peptide and incubated for 5 days. Cell proliferation was measured by the MTT method. ■, control; □, 1 \( \mu \)g; △, 2.5 \( \mu \)g; ○, 5 \( \mu \)g; △, 10 \( \mu \)g; ○, 25 \( \mu \)g of peptide-coated cells. Panel c, to determine whether the peptide adsorbed to well surface from the medium during the incubation, WM9 and WM164 cells were treated with \( \alpha_3(IV)185–203 \) peptide for 5 days. After that, cells were removed with 0.1% sodium dodecyl sulfate, and any adsorbed peptide was detected with the polyclonal antibody using ELISA as described under “Materials and Methods.” ■, control (without peptide); □, 1 \( \mu \)g; △, 2.5 \( \mu \)g; ○, 5 \( \mu \)g of peptide-treated cells. Without cells means that wells were incubated with medium containing varying amounts of the peptide in the absence of cells. After removal of the medium any adsorbed peptide was measured by ELISA. Panel d, to assess whether cell proliferation could be resumed, WM9 cells were first treated with \( \alpha_3(IV)185–203 \) (5 \( \mu \)g/ml) for 5 days (group 1). After removal of the peptide, cells were incubated with peptide-free medium for an additional 6 days (group 2). [\( ^3 \)H]Thymidine uptake by cells without peptide treatment was taken as control (100%). [\( ^3 \)H]Thymidine uptake by cells treated with peptide is presented as the percentage of control. ■, control; □, experimental. Error bars represent S.D. from the mean.
specific role the NC1 domain of the α3(IV) chain may play in vivo in the maintenance of the normal phenotype of overlying epithelial cells or of transmigrating tumor cells. Secondary structure studies have shown that the sequence, which contains the triplet -SNS- of the α3(IV) chain, occurs within one of the two β-turns occupied by the α3(IV)185–203 peptide sequence (14). It is hypothesized that the NC1 domain of the α3(IV) chain must be exposed within the basement membrane in a way that this region of the chain is on the outside and promotes contact with the transmigrating PMN or tumor cells or with overlying epithelial cells in a given tissue.

In this report we have demonstrated that a peptide from the NC1 domain of the α3(IV) chain of type IV collagen, comprising residues 185–203, contains a multifunctional domain that promotes cell adhesion and inhibits proliferation of melanoma cells. An absolute requirement for these biological activities is the presence of a triplet -SNS- within this peptide. Studies are currently under way to elucidate the steps in the transduction pathway involved in these biological activities and to isolate and characterize the putative receptor that recognizes the above peptide.

Fig. 3. Panel a, effect of anti-peptide antibodies on attachment of WM9 melanoma cells to peptides. Each well was coated with synthetic peptide α3(IV)185–203 (2.5 μg/well) overnight. Monoclonal or polyclonal antibodies to the peptide were added to the wells as described under “Materials and Methods.” Cells were seeded on the polyclonal antibody-treated (1:50 dilution) or on the monoclonal antibody A5D7-treated (undiluted) substrates (columns 2 and 3). Cells were also seeded on the peptide-containing substrate without antibody treatment as control (column 1). Monoclonal antibody D12H5 (undiluted), which reacted very weakly with all of the peptides, was also used as control antibody (column 4). The inhibitory effect of the monoclonal and polyclonal antibodies on the biological properties of the peptide is evident. Panel b, effects of anti-peptide antibodies on melanoma cell proliferation. The synthetic peptide α3(IV)185–203 was incubated with antibodies before adding to the medium. Monoclonal antibodies A5D7 and D12H5 were used at a 1:1 dilution. The polyclonal antibody was in a 1:50 dilution. After that, the antibody-treated peptides (5 μg/ml) were added to the medium, and cell proliferation was determined by the MTT method. f, control; □, α3(IV)185–203-SNS (unsubstituted); ○, α3(IV)185–203-ANS; □, α3(IV)185–203-SNA. Error bars represent S.D. from the mean.

Fig. 4. The modified α3(IV)185–203 peptides, where the serine in positions 189 or 191 was replaced by alanine, i.e. -ANS- and -SNA-, were used to identify the cell binding domain in α3(IV) peptide. Panel a, attachment of melanoma cells to the substitute peptides of α3(IV)183–203 (2.5 μg/well) where the serine was replaced by alanine. 2.5 × 10⁵ [35S]methionine-labeled cells were added to each well, and attached cells were measured as described under “Materials and Methods.” ■, control; □, α3(IV)185–203-SNS (unsubstituted); ○, α3(IV)185–203-ANS; □, α3(IV)185–203-SNA. Panel b, effects of the modified α3(IV) peptide on melanoma cell proliferation. The modified α3(IV) peptides (5 μg/ml) were added to the medium, and cell proliferation was monitored by the MTT method. ■, control; □, α3(IV)-SNS; ○, α3(IV)-SNA; , α3(IV)-ANS. Error bars represent S.D. from the mean.
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