Regulation of Tumor Cell Chemotaxis by Type IV Collagen Is Mediated by a Ca$^{2+}$-dependent Mechanism Requiring CD47 and the Integrin $\alpha_V\beta_3$*

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Studies from our laboratories demonstrated that synthetic peptides from the non-collagenous (NC-1) domain of the $\alpha_3$ (IV) chain of type IV collagen (COL IV) enhanced tumor cell adhesion (Han, J., Ohno, N., Monboisse, J. C., Pasco, S., Borel, J. P., and Kefalides, N. A. (1997) J. Biol. Chem. 272, 20395–20401). We have isolated the receptors for the $\alpha_3$(IV)185–203 peptide from melanoma and prostate tumor cells and identified them as CD47/integrin-associated protein and the integrin $\alpha_V\beta_3$ (Shahan, T. A., Ziaie, Z., Pasco, A., Bellon, G., Monboisse, J. C., and Kefalides, N. A. (1999) Cancer Res. 59, 4584–4590). In the present study we have examined the effect of CD47 and the integrin $\alpha_V\beta_3$ on in vitro tumor cell chemotaxis and Ca$^{2+}$-modulation in response to COL IV, from the anterior lens capsule (ALC-COL IV) and peptides from its NC-1 domain. COL IV as well as the $\alpha_3$(IV) peptide promoted tumor cell chemotaxis with an immediate increase in intracellular [Ca$^{2+}$]. Treating tumor cells with CD47 and integrin $\alpha_V\beta_3$-reactive antibodies reduced chemotaxis as well as the rise in [Ca$^{2+}$], in response to ALC-COL IV or the $\alpha_3$(IV)185–203 peptide but not to Engelbreth-Holm-Swarm-COL IV or fibronectin. The $\alpha_3$(IV)185–203 synthetic peptide stimulated an increase in calcium from intracellular stores exclusively, whereas ALC-COL IV, Engelbreth-Holm-Swarm-COL IV, and fibronectin stimulated Ca$^{2+}$ flux from both internal and external stores. Furthermore, treatment of the cells with Ca$^{2+}$ chelator bis-(O-aminophenoxy)ethane-N,N,N',N'-tetraacetacacid-acetomethoxy ester inhibited chemotaxis toward both ALC-COL IV and the $\alpha_3$(IV)185–203 peptide. These data indicate that CD47 and integrin $\alpha_V\beta_3$ regulate tumor cell chemotaxis in response to COL IV and the $\alpha_3$(IV)185–203 peptide through a Ca$^{2+}$-dependent mechanism.

Transmigration through the vessel wall by tumor cells requires adhesion and penetration of the basement membrane (BM), followed by movement in or out of the vessel lumen. These processes are mediated through specific adhesion molecules on tumor cells and endothelial cells (1, 2). Integrins play an important role in cellular adhesion and motility by allowing cellular adhesion and activation of signal transduction pathways (3, 4).

Normal COL IV is a triple-helical molecule formed by the interaction of any of two different a-chains (a1–a6) (5–7). We have shown that synthetic peptides comprising residues 185–203 of the NC-1 domain of the $\alpha_3$ chain of COL IV from anterior lens capsule (ALC), enhance melanoma cell adhesion by 50–60% over controls. On the other hand, peptides from a similar region of the other chains (i.e. a1, a2, a4, or a5) were less efficient in promoting adhesion. In these biological activities, the presence of the -SNS- triplet (residues 189–191) in the above peptide appears to be an absolute requirement (8). Pretreatment of the $\alpha_3$(IV)185–203 peptide substrate with a peptide-reactive monoclonal antibody (mAb) inhibited melanoma cell attachment; however, the treatment of a substrate composed of EHS-COL IV did not inhibit attachment.

More recently we have isolated the $\alpha_3$(IV)185–203 peptide-specific receptors on melanoma and prostate tumor cells and identified them as CD47/integrin-associated protein and the $\alpha_V\beta_3$ integrin by affinity chromatography and Western blot analysis (9). COL IV from the Engelbreth-Holm-Swarm (EHS) mouse tumor, which contains only the a1 and a2 chains of COL IV (10) has been previously demonstrated to induce melanoma cell chemotaxis (11). The role of CD47 in tumor cell chemotaxis has never been explored; however, Cooper et al. (12) and Parkos et al. (13) demonstrated the requirement of CD47 for normal trans-endothelial and trans-epithelial migration of PMN. Work by Parkos et al. (13) showed that PMN, pretreated with CD47-reactive antibodies at levels above 2 μg/ml, were inhibited from trans-endothelial migration in response to n-formylmethionyl-leucylphenylalanine. They concluded that the inhibition was due to a yet unknown post-adhesive event following binding to the $\beta_3$ integrin subunit.

The signal transduction pathways by which CD47 and inte-

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1 The abbreviations used are: BM, basement membrane; ALC-COL IV, type IV collagen from anterior lens capsule; BAPTA-AM, bis-(O-aminophenoxy)ethane-N,N,N',N'-tetraacetacacid-acetomethoxy ester; IAP, integrin-associated protein; COL IV, type IV collagen; EHS, Engelbreth-Holm-Swarm tumor; PBS, fetal bovine serum; HBSS, Hanks’ balanced salt solution; i, intracellular; mAb, monoclonal antibody; NC-1, noncollagenous domain; HPF, high power field; PMN, polymorphonuclear leukocyte.

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Type IV Collagen Isolation from Bovine Anterior Lens Capsule and Preparation of Its Synthetic Peptides—COL IV was extracted from bovine ALC according to the protocol of Brinker et al. (24). EHS-COL IV, which contains only the α1 and α2 chains, was a gift from Dr. Hynda Kleinman, National Institutes of Health (25). Synthetic peptides corresponding to the primary sequence regions of the NC-1 domain of human COL IV α1 and α3 chains (26) were synthesized and purified at the Protein Core Facility at the University of California, San Francisco. The OV10 cell line, which is CD47-negative, was made CD47-positive by transfection (22). The line was obtained from the American Type Culture Collection (Bethesda, MD). The melanoma cell line was cultivated in medium MCD-15 (78%) with L-15 medium (10%), trypsin phosphate broth (10%), FBS (2%), and 50 units/ml insulin as described previously by Herlyn et al. (20, 21). The prostate tumor cell line was cultivated in Eagle’s minimum essential medium with 2% FBS. The ovarian tumor cell lines (OV10), which are CD47-positive, were obtained from Dr. Eric Brown and Dr. Erny Herlyn at the University of California, San Francisco. The OV10 cell line, which is CD47-negative, was made CD47-positive by transfection (22). The line designated OV10(−) is CD47-negative and αβ3-positive, while the OV10(+)+ is CD47-positive and αβ3-negative (19). The CS-1(−) melanoma cell line, which is CD47-positive and αβ3-negative, was obtained from Dr. Caroline Damsky at the University of California, San Francisco. The OV10 cell line, which is CD47-negative, was made CD47-positive by transfection (22). The line designated OV10(−) is CD47-negative and αβ3-positive, while the OV10(+) is CD47-positive and αβ3-negative (19). The CS-1(−) melanoma cell line, which is CD47-positive and αβ3-negative, was obtained from Dr. Caroline Damsky at the University of California, San Francisco (23). The above cells were cultivated in Isocase’s modified Dulbecco’s medium, 2% FBS, and Genticin (10 μg/ml) and counted before plating using a Coulter counter (model ZM; Coulter, Hialeah, FL) or Neubauer hemocytometer.

Type IV Collagen Isolation from Bovine Anterior Lens Capsule and Preparation of Its Synthetic Peptides—COL IV was extracted from bovine ALC according to the protocol of Brinker et al. (24). EHS-COL IV, which contains only the α1 and α2 chains, was a gift from Dr. Hynda Kleinman, National Institutes of Health (25). Synthetic peptides corresponding to the primary sequence regions of the NC-1 domain of human COL IV α1 and α3 chains (26) were synthesized and purified at the Protein Core Facility at the University of Pennsylvania (Philadephia, PA) essentially according to the method of Barany and Merrifield (27). One of the peptides, α3(IV)185–203, was derived from an alternatively spliced human α3(IV) collagen clone missing exon IV as described previously (28). The peptides were solubilized in medium without FBS (overnight at 4 °C) or with 10 μl of dimethyl sulfoxide and diluted to the appropriate concentration in medium with FBS (2%). Vehicle control consisted of only medium into which peptides were diluted. Primary sequences of synthetic peptides used in this study are listed in Table I.

| Table I | Primary amino acid sequences of synthetic peptides from COL IV |
|---------|---------------------------------------------------------------|
| α3(IV)185–203 | CNYNNSYSFYLSNPER |
| α3(IV)179–208 | CHGRTCNYNNNSFYLSNPERDFRKF |
| α3(IV)190–203 | NSYSSFLSNSNPER |
| α1(IV)185–203 | CNYNAYSFWLATTER |
| α3(IV)5–15 | CQDDLALFYVNLSP |

**Chemoataxis Analysis**—Tumor cell chemotaxis toward COL IV or the α3(IV)185–203 peptide was measured using multiple 12-well chemotaxis chambers from Neuroprobe Inc. (Cabin John, MD) (29). Cells were detached from culture by incubation with Sigma cell dissociation medium and washed three times with Hank’s balanced salt solution (HBSS) with 0.3% bovine serum albumin and incubated for 1 h (37 °C at 5% CO2) in the same. In some cases, cells were pretreated for 30 min with integrin-reactive mAbs and washed (twice) with HBSS prior to their addition to the upper well. In other studies, cells were pretreated with BAPTA-AM (145 μM) for 25 min at 37 °C or dotarazine (10 μM) or nifedipine (50 μM), Ca2+ channel modulators, for 60 min before use. Chemotactic assays were performed using a Leukostat kit (Fisher Scientific Inc.), and mounted on glass microscope slides according to manufacturer’s instructions. Motility was analyzed by manually counting 6 high power oil-immersion fields using an Olympus microscope (model BH-2) equipped with a Toshiba CCD color video camera (model IXTU40a, Laser and Motion Development, Union City, CA) connected to a PC running Image tool software for Windows (University of Texas, version 1.28).

**Measurement of Intracellular [Ca2+] by Fura-2 in Melanoma Cells**—To determine if COL IV and synthetic peptides stimulate a rise in [Ca2+]i, fura-2 fluorescence was measured in melanoma cells. Cells were removed from adherence by incubation with cell dissociation media, washed twice in HBSS without phenol red, and placed in the same supplemented with 0.1% bovine serum albumin. Fura-2-acetoxymethyl ester was solubilized in MeSO (99%) and added to the cell suspension (1.67 × 106/ml) at a final concentration of 25 μM and incubated for 15 min at 37 °C, washed and resuspended in Ca2+-free HBSS containing 1 mM EGTA, and used immediately. For extracellular Ca2+ depletion studies, cells were incubated in Ca2+-free HBSS containing 1 mM EGTA for 45 min at 4 °C. For intracellular depletion studies, cells were incubated in Ca2+-free HBSS containing BAPTA-AM (145 μM) for 20 min. In these experiments, COL IV or its synthetic peptides were added to cells alone or to cells that had been previously treated with integrin-reactive mAbs (30 min) and washed (twice) prior to their addition.

Fura-2 fluorescence was measured using a Photon Technologies Inc. (South Brunswick, NJ) fluorescent spectrophotometer (model MP-2) equipped with a mercury arc lamp and a Windows-based PC running PFI Alpha Scan software (version 2.050). Incubation temperature was automatically maintained at 37 °C. Software was set to allow alternating excitation wavelengths at 340 and 380 nm every 2 s while emission spectra passed through a 510-nm filter before measurement with a photon detector. Uptake of Fura-2 AM among cells was uniform, as determined by fluorescence microscopy immediately after loading and before (1 and 2 h after loading (30)).

**Calibration of the Fura-2 Signal in Fura-2-loaded Melanoma Cells**—Melanoma cells were washed twice and resuspended in Ca2+- and Mg2+-free HBSS with 25 μM ionomycin and 7 mM EGTA. To the cell suspension, an equal volume of HBSS containing 5 mM EGTA, 1 mM Mg2+, and 20 μM ionomycin was added. Free [Ca2+]i concentrations were calculated using the equation of Grynkiewicz (30). Data were then expressed as nanomolar Ca2+.

**Statistical Analysis**—Data were analyzed using Sigmaplot (Jandel Scientific Software, San Rafael, CA) and were expressed as mean ± S.E. To evaluate the effect of the receptors on agent-mediated chemotaxis, comparisons were performed using Student’s t test. Data were considered statistically significant if p values were <0.05 (31).

**RESULTS**

**The Effect of COL IV and Its Synthetic Peptides on Tumor Cell Chemotaxis**—Tumor cell chemotaxis in response to COL IV...
Motility toward fibronectin peaked at 100 µm COL IV, or the effect on tumor cell chemotaxis in response to ALC-COL IV, EHS-COL IV cells/HPF. Determinations for four experiments expressed as the number of subtracted out for each data point. Data represent the mean of triplicate (in the bottom well) as described. Nonspecific random migration was failed to induce chemotaxis; similarly, cells responded poorly to the α1(IV)185–203 peptide (which contains -NS-, rather than the -SNS- sequence) failed to induce chemotaxis; similarly, cells responded poorly to the α1(IV)185–203 peptide, which has the -ANS- sequence, even at 1000 µg/ml. Chemotaxis of melanoma cells toward ALC-COL IV peaked at approximately 10 µg/ml at 533 ± 41 cells/HPF, toward EHS-COL IV peaked at 1000 µg/ml with 332 ± 31 cells/HPF and toward the α3(IV)185–203 synthetic peptide peaked at 10 µg/ml with 373 ± 30 cells/HPF (Fig. 1). Motility toward fibronectin peaked at 100 µg/ml with 213 ± 24 cells/HPF for fibronectin (data not shown). It is evident that chemotaxis of melanoma cells toward ALC-COL IV and its α3(IV) peptides was more efficient as compared with EHS-COL IV or synthetic peptides lacking the -SNS- tripeptid.

The Effect of CD47 and integrin αβ3 reactive antibodies on melanoma cell chemotaxis in response to COL IV and its synthetic peptides—We have recently isolated the cellular receptors from both melanoma and prostate tumor cells for the α3(IV)185–203 peptide ligand and identified them as CD47 and the αβ3 integrin (9). To determine the effect of these receptors on tumor cell chemotaxis in response to ALC-COL IV, EHS-COL IV, or the α3(IV)185–203 peptide (10 µg/ml), cells were pretreated with receptor-reactive antibodies before analysis (Fig. 2A and B). Treatment of melanoma cells with the CD47-reactive mAb (1 µg/ml) inhibited chemotaxis toward ALC-COL IV by 5% (509 ± 55 cells/HPF) (Fig. 2A), toward the α3(IV)185–203 peptide by 16% (275 ± 33 cells/HPF) (Fig. 2B) and had no effect on chemotaxis toward EHS-COL IV (182 ± 23 cells/HPF) (data not shown). Treatment of the melanoma cells with the αβ3 integrin-reactive mAb (0.5 µg/ml) inhibited chemotaxis toward ALC-COL IV by 24% (403 ± 34 cells/HPF) (Fig. 2A), toward the α3(IV)185–203 peptide by 32% (221 ± 27 cells/HPF) (Fig. 2B) and had no effect toward EHS-COL IV (188 ± 39 cells/HPF) (data not shown). Treatment of cells with both mAbs together inhibited chemotaxis toward the ALC-COL IV by 34% (323 ± 33 cells/HPF) (Fig. 2A) and toward the α3(IV)185–203 peptide by 71% (103 ± 22 cells/HPF) (Fig. 2B). As a control, β1 and β2 integrin subunit-reactive mAbs (2 µg/ml) were also tested. These antibodies had no effect on chemotaxis toward the α3(IV)185–203 peptide; however, they inhibited chemotaxis toward ALC-COL IV by 23% and 6%, respectively (data not shown). This is consistent with previous observations, which demonstrate that COL IV is a ligand for the β1 and β2 integrin subunits (32, 33).
The Effect of CD47 and αvβ3 Integrin Expression on Tumor Cell Chemotaxis in Response to COL IV and Its Synthetic Peptides—To further test the effect of these receptors on chemotaxis toward ALC-COL IV and the peptides, we examined the ability of the ovarian tumor cell lines, OV10(+) (CD47-positive) and the OV10(−) (CD47-negative), as well as the CS-1(−) (integrin β3-negative) melanoma cell line, to respond to the chemotacticants. Chemotaxis of the OV10(+) cell line toward ALC-COL IV at 100 μg/ml was 441 ± 23 cells/HPF, whereas chemotaxis of the OV10(−) cell line was only 341 ± 28 cells/HPF (Fig. 3A). In comparison, chemotaxis of the CS-1(−) cell line in response to ALC-COL IV at 100 μg/ml was 98 ± 4 cells/HPF in the CS-1(−) cell line (Fig. 3A). To determine the influence of CD47 and the integrin αvβ3 receptors in chemotaxis toward ALC-COL IV, tumor cells were treated with receptor blocking mAbs before analysis. Treatment of the OV10(+) cell line with CD47-reactive mAb inhibited chemotaxis by 6% (433 ± 34 cells/HPF), whereas treatment with the αvβ3 integrin-reactive mAb inhibited chemotaxis by 21% (367 ± 31 cells/HPF) toward ALC-COL IV (100 μg/ml) (Fig. 2A). Treatment of the CS-1(−) cell line with CD47-reactive mAb inhibited chemotaxis by 4% (92 ± 12 cells/HPF), whereas treatment with the αvβ3 integrin-reactive mAb had no effect on chemotaxis in response to ALC-COL IV (Fig. 2A).

When the α3(IV)185–203 peptide (100 μg/ml) was used as a chemoattractant, chemotaxis of the OV10(+) cell line was 192 ± 13 cells/HPF, whereas of the OV10(−) cell line it was rather lower (98 ± 11 cells/HPF) at the same concentration (Fig. 3B). In comparison, chemotaxis of the CS-1(−) cell line in response to α3(IV)185–203 peptide at 100 μg/ml was 51 ± 4 cells/HPF in the CS-1(−) cell line. To determine the role of CD47 and integrin αvβ3 in chemotaxis toward the α3(IV)185–203 peptide, tumor cells were treated with receptor blocking mAbs before analysis (Fig. 2B). Treatment of the OV10(+) cell line with the CD47-reactive mAb (1 μg/ml) inhibited chemotaxis by 13% (167 ± 17 cells/HPF), whereas treatment with αvβ3 integrin-reactive mAb (0.5 μg/ml) inhibited chemotaxis by 63% (70 ± 11 cells/HPF) toward the α3(IV)185–203 peptide (100 μg/ml). On the other hand, treatment with both antibodies inhibited chemotaxis by 82% (32 ± 6 cells/HPF) toward the α3(IV)185–203 peptide. Fig. 2B shows that treatment of the OV10(−) cell line (lacking the CD47 receptor) with the CD47-reactive mAb failed to effect chemotaxis (114 ± 11 cells/HPF), whereas treatment with αvβ3 integrin-reactive mAb inhibited chemotaxis by 39% (70 ± 11 cells/HPF) toward the α3(IV)185–203 peptide (100 μg/ml). As expected, treatment with both antibodies inhibited chemotaxis at the same level as treatment with the αvβ3 integrin-reactive mAb alone. Treatment of the CS-1(−) cell line (lacking the αvβ3 receptor) with the CD47-reactive mAb inhibited chemotaxis by 47% (24 ± 8 cells/HPF), whereas treatment with the αvβ3 integrin-reactive mAb had no effect on chemotaxis in response to the α3(IV)185–203 peptide (Fig. 2B). Together, these data indicate that both CD47 and the αvβ3 integrin are needed for normal chemotaxis toward ALC-COL IV, EHS-COL IV, and the α3(IV)185–203 peptide; however, the αvβ3 integrin plays a more substantial role than CD47 in chemotaxis toward these ligands.

The Effect of Calcium Modulation on Melanoma Cell Chemotaxis in Response to COL IV and Its Synthetic Peptides—To investigate the effect of Ca2+ flux on melanoma cell chemotaxis in response to COL IV and its peptides, cells were pretreated with the Ca2+ chelator BAPTA-AM (145 μM) or with the Ca2+ channel modulators dantazol (inhibits only Ca2+ channel types) (10 μM) or nifedipine (inhibits L-type Ca2+ channels) (50 μM). Pretreatment of the melanoma cells with BAPTA-AM (145 μM) inhibited chemotaxis by 81% toward ALC-COL IV (89 ± 10 cells/HPF), by 79% toward EHS-COL IV (52 ± 7 cells/HPF), and by 95% toward the α3(IV)185–203 peptide (15 ± 6 cells/HPF) (Fig. 4); these results indicate the general need for Ca2+ in melanoma cell chemotaxis. Similar experiments, performed without external Ca2+, demonstrated that chemotaxis toward the α3(IV)185–203 peptide proceeded at near normal levels; however, chemotaxis toward COL IV or fibronectin was inhibited (data not shown). Addition of Ca2+ to the cell medium returned melanoma cell chemotaxis toward ALC-COL IV and EHS-COL IV to near normal levels; however, it had little effect on chemotaxis toward the α3(IV)185–203 peptide (data not shown). Pretreatment of melanoma cells with dantazol (10 μM) inhibited chemotaxis by 62% toward ALC-COL IV (197 ± 21 cells/HPF) and by 72% toward EHS-COL IV (77 ± 13 cells/HPF), but this was not observed with nifedipine treatment.

FIG. 3. The effect of ALC-COL IV or the α3(IV)185–203 peptide on tumor cell chemotaxis. Tumor cell chemotaxis toward increasing concentrations of either ALC-COL IV (A) or the α3(IV)185–203 peptide (B) was tested. Melanoma cells (W-164, •), prostate tumor cells (DU-145, ○), ovarian tumor cells OV10(+) (▴) (expresses both CD47 and αvβ3 integrin), OV10(−) (□) (expresses only integrin αvβ3), and the CS-1 melanoma cell line (■), which expresses CD47 but not integrin αvβ3, were tested. Non-specific random migration was subtracted out for each data point. Data represent the mean of triplicate determinations for four experiments (± S.E.) expressed as the number of cells/high power field. ∗, p < 0.05 as compared with agent alone.
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**Fig. 4. The effect of intracellular Ca^{2+} modulation on melanoma cell chemotaxis in response to ALC-COL IV, EHS-COL IV, and the α3(IV)185–203 peptide.** To determine the effect of Ca^{2+} on melanoma cell (W-164) chemotaxis toward either ALC-COL IV (closed bars), EHS-COL IV (cross-hatched bars) or the α3(IV)185–203 peptide (open bars). Cells were treated with either the Ca^{2+} channel modulators dotarazine or nifedipine, or the Ca^{2+} chelator BAPTA-AM (20 min at RT) before addition to the upper well of a modified Boyden chamber. Chemotaxis was analyzed by modified Boyden chamber as described in Fig. 1. All chemoattractants were used at 100 μg/ml. Nonspecific random migration was subtracted out for each data point. Data represent the mean of triplicate determinations for one experiment (± S.E.) expressed as the number of cells/HPF.

**Calreticulin Modulation in Response to ALC-, EHS-COL IV, and Synthetic Peptides in Melanoma Cells—**We have shown that Ca^{2+} channel modulators as well as the Ca^{2+} chelator BAPTA-AM influence chemotaxis toward COL IV and its synthetic peptides (Fig. 4). To further understand the effect of COL IV and the α3(IV)185–203 peptide as well as the role of CD47 and α_{3}β_{3} integrin receptors on [Ca^{2+}], levels in tumor cells, we measured actual [Ca^{2+}] levels in melanoma cells in response to these agents in the presence or absence of receptor-reactive mAbs or dotarazine. ALC-COL IV, EHS-COL IV and the α3(IV)185–203 (100 μg/ml) caused an immediate rise in [Ca^{2+}] in the melanoma cell line (W-164) of 1271 ± 69 nM, 1115 ± 75 nM, and 1103 ± 34 nM (n = 3), respectively (Fig. 5A). By comparison, the α3(IV)185–203, α2(IV)185–203, and α3(IV)190–203 synthetic peptides, which lack the -SNS-sequence, had little effect on [Ca^{2+}], (Fig. 5A). Basal melanoma cell cytoplasmic [Ca^{2+}] level for the melanoma cell line W-164 was calculated to be 195 ± 37 nM on average.

Treatment of melanoma cells with the CD47 or the α_{3}β_{3} integrin-reactive mAbs decreased [Ca^{2+}], and therefore mobilization in response to ALC-COL IV and the α3(IV)185–203 peptide as compared with levels without antibody treatment. When melanoma cells were treated with the CD47 mAb (1 μg/ml), [Ca^{2+}] decreased primarily in response to the α3(IV) peptide; mobilization peaked at 1068 ± 25 nM in response to ALC-COL IV, 1124 ± 59 nM in response to EHS-COL IV, and 623 ± 13 nM in response to the α3(IV)185–203 peptide. Treatment of melanoma cells with the α_{3}β_{3} integrin-reactive mAb allowed [Ca^{2+}] to peak at 791 ± 26 nM in response to ALC-COL IV, 1121 ± 94 nM in response to EHS-COL IV, and 701 ± 9 nM in response to the α3(IV)185–203 peptide. Whereas treatment of the tumor cells with both mAbs together decreased [Ca^{2+}], mobilization to 0 nM in response to the α3(IV)185–203 peptide, it allowed [Ca^{2+}], to peak at 797 ± 57 nM in response to ALC-COL IV and 1127 ± 107 nM in response to EHS-COL IV (Fig. 5B). Treatment of the cells with the CD47 mAb at levels above 10 μg/ml inhibited Ca^{2+} mobilization in response to both EHS-COL IV or fibronectin (data not shown), indicating that high levels of the CD47-reactive mAb may inhibit Ca^{2+}, mobilization in a nonspecific manner. Comparison of these data to studies without antibodies was made using the data presented in Fig. 5A. As an additional control, the cells were treated with either integrin β_{1} or β_{3} subunit-reactive mAbs. These mAbs had little effect on [Ca^{2+}], at the concentration used. Treatment of the cells with the antibodies alone had no effect on resting [Ca^{2+}], (data not shown). These data indicate that the α3(IV)185–203 peptide initiates Ca^{2+} mobilization by virtue of the -SNS- at residues 189–191; they also demonstrate that both CD47 and the α_{3}β_{3} integrin regulate Ca^{2+}, mobilization in response to the α3(IV)185–203 peptide, ALC-COL IV, and EHS-COL IV.

To determine whether the α3(IV)185–203 peptide stimulated Ca^{2+} flux was from internal or external sources, specific channel modulators were used. The calcium channel modulator dotarazine (Fig. 5B) had no effect on Ca^{2+} mobilization, with (Fig. 5B) or without (data not shown) external Ca^{2+} on melanoma cells stimulated with the α3(IV)185–203 synthetic peptide, further suggesting that the primary source of Ca^{2+} is from intracellular stores. In contrast, ALC-COL IV or EHS-COL IV failed to increase [Ca^{2+}], in the presence of dotarazine (Fig. 5B). Treatment with nifedipine had no effect (data not shown) on [Ca^{2+}], mobilization. As expected, treatment of cells with BAPTA-AM inhibited Ca^{2+}, mobilization in response to all agents (data not shown).

**DISCUSSION**

The ability of CD47 and integrin α_{3}β_{3} to function as receptors mediating chemotaxis in response to the α3 chain of COL IV is a novel observation. Type IV collagen is found in BM and contributes to its structural and functional properties. Our studies have discovered additional biological properties of BMs that can be attributed to COL IV. Because of their critical location, underlying all endothelial and epithelial surfaces, BMs can be considered as our first line of defense. As with the enhancement of PMN chemotaxis toward BM components (32), the present studies demonstrate that COL IV and the α3(IV)185–203 peptide enhance tumor cell chemotaxis. The chemotactic potential of other BM components such as fibronectin, laminin, and entactin has also been demonstrated (34, 35).

In previous studies, we demonstrated that the α3(IV)185–203 peptide bound specifically to CD47 and the α_{3}β_{3} integrin (9). CD47 is known to influence PMN motility; however, the mechanism of this process is unknown (12, 13). In this report, we present evidence which demonstrates that these receptors are necessary for unabated tumor cell chemotaxis toward COL IV and the α3(IV)185–203 peptide. Treatment of these cells with either CD47 or integrin α_{3}β_{3} mAbs alone partially inhibited chemotaxis; however, treatment of the tumor cells with both mAbs together markedly inhibited chemotaxis toward the α3(IV)185–203 peptide and ALC-COL IV. On the other hand, the mAbs had little or no effect in the presence of other chemotactants such as EHS-COL IV or fibronectin. These data indicate that CD47 and integrin α_{3}β_{3} are involved in chemotaxis in response to ALC-COL IV and the α3(IV)185–203 peptide.

CD47 has been previously shown to associate with and modulate integrin activation. Data support its association with the β_{1} (33), β_{3} (34, 35), and β_{3} (36–38) integrin subunits. Previous data from our laboratory suggest that the interaction of CD47
with \( \alpha_\beta_3 \) is necessary for the complete inhibition of tumor cell proliferation and normal tumor cell adhesion to a substrate composed of the \( \alpha_3(IV)185–203 \) peptide (9). In the present study, we have presented evidence that both these receptors are also necessary for normal chemotaxis toward the \( \alpha_3(IV)185–203 \) peptide. The same argument holds true for the rise in \([Ca^{2+}]\), in response to the \( \alpha_3(IV)185–203 \) peptide. Our current findings also demonstrate that the receptors for the \( \alpha_3(IV)185–203 \) peptide are \( \alpha_\beta_3 \)-reactive mAbs alone inhibited the rise in \([Ca^{2+}]\), (Fig. 5, A and B). In comparison, treatment of melanoma cells with either CD47 or integrin \( \alpha_\beta_3 \)-reactive mAbs alone inhibited chemotaxis partially in response to ALC-COL IV and markedly in response to the \( \alpha_3(IV)185–203 \) peptide but had no effect on chemotaxis toward EHS-COL IV. These data suggest that the rise in \([Ca^{2+}]\), in response to the \( \alpha_3(IV)185–203 \) peptide is mediated through the same receptors for the peptide as those required for chemotaxis.

Based on the predicted structure of CD47 (5 transmembrane domains) and its primary sequence homology to known channel proteins, it has been hypothesized that the receptor may function in the capacity of an ion channel or be closely associated with one (11). To further investigate the relationship among these factors, namely the peptide, the two receptors, and \( Ca^{2+} \) modulation, we examined the source of the \( Ca^{2+} \) flux. We found that the rise in \([Ca^{2+}]\), in response to the \( \alpha_3(IV)185–203 \) peptide was primarily from intracellular sources. In comparison, the rise in \([Ca^{2+}]\), in response to ALC-COL IV and EHS-COL IV was mostly from extracellular sources. Since we have demonstrated that the receptors for the \( \alpha_3(IV)185–203 \) peptide are CD47 and integrin \( \alpha_\beta_3 \), these data suggest that CD47 may not act as an ion channel in melanoma cells in response to the \( \alpha_3(IV)185–203 \) peptide; however, the receptors are likely linked to a signal transduction pathway that leads to the release of intracellular \( Ca^{2+} \) stores. On the other hand, treatment of melanoma cells with the CD47 mAb (B6H12) at concentrations above 10 \( \mu \)g/ml inhibited the rise in \([Ca^{2+}]\), in response to either EHS-COL IV and fibronectin, each of which were shown to cause a rise in \([Ca^{2+}]\), mostly from extracellular stores. This indicates that CD47 may function as a \( Ca^{2+} \) channel in response to certain agents, but not in response to the \( \alpha_3(IV)185–203 \) peptide.

In this report we have demonstrated a regulatory effect of normal type IV collagen from ALC and peptides derived from the NC-1 domain of the \( \alpha_3(IV) \) chain on tumor cell chemotaxis in vitro. Specifically, we have shown that CD47 and the \( \alpha_\beta_3 \) integrin are necessary for melanoma cell chemotaxis in response to the \( \alpha_3(IV)185–203 \) peptide. Our current findings also
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indicate that calcium flux is an important regulator of chemotaxis toward these ligands and suggest the presence of a Ca\(^{2+}\)-sensitive signal transduction pathway through CD47 and the integrin \(\alpha_v\beta_3\).

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REFERENCES
1. Oura, E. B., Sandig, M., and Siu, C. H. (1998) Microsc. Res. Tech. 43, 265–275
2. Akiyama, S. K., Olden, K., and Yamada, K. M. (1996) Cancer Metastasis Rev. 14, 173–189
3. Hynes, R. O. (1992) Cell 69, 11–25
4. Albecka, S. M., and Buck, C. A. (1990) FASEB J. 4, 2868–80
5. Gunwar, S., Saus, J., Noellken, M. E., and Hudson, B. G. (1990) J. Biol. Chem. 265, 5466–5469
6. Hudson, B. G., Reeder, S. T., and Tryggvason, K. (1993) J. Biol. Chem. 268, 26035–36
7. Leinonen, A., Mariyana, M., Mochizuki, T., Tryggvason, K., and Reeder, S. T. (1994) J. Biol. Chem. 269, 26172–26177
8. Han, J., Ohno, N., Monboisse, J. C., Pasci, S., Borel, J. P., and Kefalides, N. A. (1997) J. Biol. Chem. 272, 20385–20401
9. Shaham, T. A., Ziaie, Z., Pasco, J., Borel, J. P., and Kefalides, N. A. (1999) Cancer Res. 59, 4584–4589
10. Wisdom, B. J. Jr., Gunwar, S., Hudson, M. D., Noellken, M. E., and Hudson, B. G. (1992) Connect. Tiss. Res. 27, 225–234
11. Savarese, D. M., Russell, J. T., Fatatis, A., and Liotta, L. (1992) J. Biol. Chem. 267, 21928–21935
12. Cooper, D., Lindberg, F. P., Gamble, J. R., Brown, E. J., and Vadas, M. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3978–3982
13. Parkos, C. A., Colgan, S. P., Liang, T. W., Nusrat, A., Barcarra, A. E., Carnes, D. K., and Madara, J. L. (1996) J. Cell Biol. 132, 437–450
14. Schwartz, M. A., Brown, E. J., and Fazeli, B. (1993) J. Biol. Chem. 268, 19931–19934
15. Smith, T. W., Menter, D. G., Nicholson, G. L., and McIntire, L. V. (1996) Melanoma Res. 6, 351–362
16. Cunningham, C. C., Gorlin, J. B., Kwaizkowski, D. J., Hartwig, J. H., Janmey, P. A., Byers, H. R., and Stossel, T. P. (1992) Science 255, 325–327
17. Tejerina, T., Chulia, T., and Gonzalez, P. (1993) Eur. J. Pharmacol. 239, 75–81
18. Godfraind, T., Miller, R., and Wibo, M. (1986) Pharmacol. Rev. 38, 321–416
19. Brown, E., Hooper, L., Ho, T., and Gresham, H. (1990) J. Cell Biol. 111, 2785–2794
20. Herlyn, M., Kath, R., Williams, N., Valyi-Nagy, I., and Rodeck, U. (1990) Adv. Cancer Res. 54, 213–234
21. Herlyn, M. (1990) Cancer Metastasis Rev. 9, 101–112
22. Lindberg, F. P., Gresham, H. D., Reinhold, M. I., and Brown, E. J. (1996) J. Cell Biol. 134, 1313–1322
23. Filardo, E. J., Brooks, P. C., Deming, S. L., Damsky, C., and Cheros, D. A. (1995) J. Cell Biol. 130, 441–450
24. Brinker, J. M., Pegg, M. T., Howard, P. S., and Kefalides, N. A. (1985) Collagen Relat. Res. 5, 233–244
25. Kleinman, H. K., McGarvery, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K., and Martin, R. G. (1992) Biochemistry 21, 6188–6193
26. Kefalides, N. A., Ohno, N., Wilson, C. E., Fillit, H., Zabriski, J., and Rosenbloom, J. (1994) Kidney Int. 43, 94–100
27. Barany, G., and Merrifield, R. B. (1980) in The Peptides (Gross, E., Meinhofer, J., eds) Vol. II, pp. 1–284, Academic Press, New York
28. Feng, L., Xia, Y., and Wilson, C. B. (1994) J. Biol. Chem. 269, 2342–2348
29. Falk, W., Goodwin, R. H., Jr., and Lenard, E. J. (1988) J. Immun. Methods 33, 33239–33247
30. Grynkiewitz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
31. Dowdy, S., and Wearden, S. (1983) in Statistics for Research (Bradley, R. A., Hunter, J. S., Kendall, D. G., Miller, R. G., and Watson, G. S., eds) pp. 32–38, John Wiley & Sons, New York
32. Eble, J. A., Ries, A., Liech, A., Mann, K., Stanton, H., Gavrilovic, J., Murphy, G., and Kuhn, K. (1996) J. Biol. Chem. 271, 30964–30970
33. Vandenbrouke, P., Kern, A., Ries, A., Luckenholt-Edwards, L., Mann, K., and Kuhn, K. (1991) J. Cell Biol. 113, 1475–1483
34. Senior, R. M., Gresham, H. D., Griffin, G. L., and Chung, A. E. (1992) J. Clin. Invest. 90, 2251–2257
35. Klonies, J., Sumitran-Karuppan, S., and Hauzenberger, D. (1997) Int. J. Can. 72, 1034–1044
36. Wang, X. Q., and Frazier, W. A. (1998) Mol. Biol. Cell 9, 865–874
37. Van Strijp, J. A., Russell, D. G., Tuomanen, E., Brown, E. J., and Wright, S. D. (1993) J. Immunol. 151, 3324–3327
38. Ishibashi, Y., Claus, S., and Relman, D. A. (1994) J. Exp. Med. 180, 1225–1233
Regulation of Tumor Cell Chemotaxis by Type IV Collagen Is Mediated by a Ca\textsuperscript{2+}-dependent Mechanism Requiring CD47 and the Integrin \(\alpha\text{V}\beta\text{3}\)

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