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ADAM-15/Metargidin Mediates Homotypic Aggregation of Human T Lymphocytes and Heterotypic Interactions of T Lymphocytes with Intestinal Epithelial Cells*

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Intestinal epithelial cells (IEC) play an immunoregulatory role in the intestine. This role involves cell-cell interactions with intraepithelial lymphocytes that may also play a role in some enteropathies. The discovery of the RGD motif-containing protein ADAM-15 (a disintegrin and metalloprotease-15) raises the question of its involvement in these cell-cell interactions. Cell adhesion assays were performed using the Jurkat E6.1 T cell line as a model of T lymphocytes and Caco2-BBE monolayers as a model of intestinal epithelia. Our results show that an anti-ADAM-15 ectodomain antibody inhibited the attachment of Jurkat cells on Caco2-BBE monolayers. Overexpression of ADAM-15 in Caco2-BBE cells enhanced Jurkat cell binding, and overexpression of ADAM-15 in Jurkat cells enhanced their aggregation. Mutagenesis experiments showed that both the mutation of ADAM-15 RGD domain or the deletion of its cytoplasmic tail decreased these cell-cell interactions. Moreover, wound-healing experiments showed that epithelial ADAM-15-mediated Jurkat cell adhesion to Caco2-BBE cells enhances the mechanisms of wound repair. We also found that ADAM-15-mediated aggregation of Jurkat cells increases the expression of tumor necrosis factor- α mRNA. These results demonstrate the following: 1) ADAM-15 is involved in heterotypic adhesion of intraepithelial lymphocytes to IEC as well as in homotypic aggregation of T cells; 2) both the RGD motif and the cytoplasmic tail of ADAM-15 are involved for these cell-cell interactions; and 3) ADAM-15-mediated cell-cell interactions are involved in mechanisms of epithelial restitution and production of pro-inflammatory mediators. Altogether these findings point to ADAM-15 as a possible therapeutic target for prevention of inappropriate T cell activation involved in some pathologies.

Intestinal epithelial cells (IEC)³ play an important role for the immune response within the intestinal tract. This immunoregulatory role of IEC is mediated through interactions with intestinal intraepithelial lymphocytes. The interactions between IEC and intraepithelial lymphocytes are thought to mediate T cell recruitment to intestinal epithelia (1). In particular, cell-to-cell interactions are believed to stabilize the retention of lymphocytes within intestinal epithelia (2), thus leading to the formation of an immune barrier against intestinal pathogens. However, aberrant cell adhesion has been implicated in the pathogenesis of a number of inflammatory disorders, including inflammatory bowel disease (IBD). Among immune cells recruited during intestinal inflammation, T lymphocytes may play a role in the pathogenesis of various intestinal diseases including Crohn disease and ulcerative colitis. Indeed some intestinal diseases show increased intraepithelial lymphocytes (*i.e.* celiac disease, lymphocytic colitis, tropical sprue, and giardiasis) and/or phenotypic alteration of intraepithelial lymphocytes (celiac disease, Crohn disease, and ulcerative colitis) (3–11). Most descriptions of early Crohn disease lesions include mucosal lymphoid aggregates (12–16) that are mainly composed of T cells and that may contribute to the pathogenesis of Crohn disease (15). Thus, it is interesting to consider the cell-cell interactions involved in IBD and possible strategies for therapeutic modulation.

Only a few molecular interactions have been identified that are involved in the specific retention of lymphocytes in intestinal epithelia. It has been shown that adherence of T lymphocytes to human epithelial cell lines can be mediated through LFA-1 and ICAM-1 (17–19). CD1d, expressed on IEC, mediates T cell-IEC interactions (20). Cell-cell and cell-fusion protein adhesion assays showed that $\alpha E\beta 7$ integrin expressed on T cells mediates T lymphocytes/IEC adhesion via E-cadherin (2, 21–26). N-cadherin has also been shown to be involved in the heterotypic adhesion of malignant T cells to epithelia (27) as well as in T cell homotypic adhesion (28).

The ADAM (a disintegrin and metalloprotease) proteins, also called MDC (metalloprotease/disintegrin/Cys-rich), are a family of membrane-anchored glycoproteins that present

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³ The abbreviations used are: IEC, intestinal epithelial cells; IBD, inflammatory bowel disease; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5 (and-6) carboxyfluorescein, acetoxyethyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Vect, vector.

structural similarities with snake venom metalloproteases (29, 30). Among the 34 ADAMs described so far, human ADAM-15 is the only one that has an RGD integrin-binding motif (31), suggesting a role of ADAM-15 in integrin binding and therefore in cell-cell interactions. It has indeed been shown that the disintegrin domain of ADAM-15 interacts with $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins in an RGD-dependent manner (32, 33) and with $\alpha 9\beta 1$ independently of the RGD motif (34). In addition, overexpression of ADAM-15 in the fibroblastic cell line NIH3T3 has been found to enhance cell-cell interactions (35).

ADAM-15 has also been shown to be expressed in human peripheral blood lymphocytes as well as human hematopoietic cell lines, including Jurkat cells (31, 36, 37). We previously reported that human IEC express ADAM-15 on the cell membrane surface (38), and very recently we also found that during IBD ADAM-15 is strongly up-regulated and ADAM-15-positive IEC are in close contact with $\alpha 5\beta 1$ integrin-positive leukocytes (39). These findings suggest a role for ADAM-15 in leukocyte transepithelial migration that occurs during IBD. Our aim was therefore to investigate whether ADAM-15 participates in cell-cell interactions between IEC and T lymphocytes.

MATERIALS AND METHODS

Cell Lines and Cell Culture—The Caco2-BBE cell line (40–43) that differentiates at confluency into enterocyte-like cells was generously provided by Dr. M. S. Mooseker. The human leukemic T cell line Jurkat E6.1 was obtained from the American Type Culture Collection (ATCC). Caco2-BBE cells were grown in high glucose Dulbecco's Vogt modified Eagle's medium (Invitrogen) supplemented with 14 mmol/liter NaHCO_3 , 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), and 1.5 $\mu\text{g}/\text{ml}$ plasmocin (Invivogen, San Diego, CA). Transfected Caco2-BBE cells were maintained in the same medium containing 1.2 mg/ml G418 (Invitrogen). Jurkat E6.1 cells were grown in RPMI medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen) and 1.5 $\mu\text{g}/\text{ml}$ plasmocin (Invivogen). Transfected Jurkat cells were maintained in the same medium containing 1 mg/ml G418 (Invitrogen). Cells were kept at 37 °C, in 5% CO_2 , and 90% humidity.

For adhesion assays with Jurkat cells, Caco2-BBE cells were plated on 12-well plates (Costar, VWR, Suwanee, GA), and experiments were performed post-confluency. For aggregation experiments, Jurkat cells were seeded in 96-well plates (Costar) to a density of 10^5 cells per 100 μl per well.

Normal Human Peripheral Blood Mononuclear Cell Isolation—Human peripheral blood mononuclear cells were isolated as follow. Whole blood was diluted 1:1 with PBS and centrifuged ($400 \times g$, 25 min, 21 °C) over Histopaque 1077 (Sigma). Mononuclear cells were aspirated and washed in PBS ($200 \times g$, 10 min, 21 °C). Cells were then lysed to be processed for Western blot experiments.

Western Blot Analysis—For total protein extraction, cells or tissues were lysed for 30 min at 4 °C in RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.1% Nonidet P-40) supplemented with protease inhibitors (Roche Diagnostics). The homogenates were centrifuged at $13,000 \times g$ for 30 min at 4 °C, and the supernatants were col-

lected for Western blot analysis. Protein concentrations were determined using the Folin assay (DC protein assay kit, Bio-Rad). Protein extracts were mixed in Tricine sample buffer (Bio-Rad), boiled for 5 min, run on a 7.5% (w/v) polyacrylamide gel (Bio-Rad) or an 8% (w/v) polyacrylamide gel (VWR, Suwanee, GA), and then transferred to nitrocellulose membranes. Membranes were blocked overnight at 4 °C or for 1 h at room temperature with 5% nonfat milk in blocking buffer and then incubated 1 h at room temperature with a rabbit polyclonal antibody raised against the cytoplasmic tail of human ADAM-15 (1:1,000; R&D Systems, Minneapolis, MN) or with a mouse monoclonal antibody raised against the viral tag protein V5 (1:1,000; Invitrogen). After washing, membranes were further incubated 1 h at room temperature with an anti-rabbit horseradish peroxidase-conjugated antibody (1:1,000; Amersham Biosciences). Membranes were washed again, and immunoreactive proteins were detected on film (Denville, Metuchen, NJ) using an enhanced chemiluminescence (ECL) substrate according to the manufacturer's instructions (Amersham Biosciences).

Membrane Preparations—For membrane preparations, cells were washed twice in PBS. After centrifugation ($400 \times g$ for 5 min), cell pellet was resuspended and carefully homogenized, with a Douncer, in HEPES (5 mM) containing protease inhibitors, incubated for 30 min at 4 °C, and then centrifuged at $13,000 \times g$ at 4 °C for 30 min. The resulting pellet was suspended in PBS by repeated passage through an 18-gauge needle. Protein concentration in the membrane suspension and in total extracts was quantified with the Bio-Rad protein assay (Bio-Rad).

Caco2-BBE Full-length ADAM-15 cDNA Cloning and Plasmid Construction—Poly(A)⁺ RNA from Caco2-BBE was isolated with a Micro Fast Track kit (Invitrogen) according to the manufacturer's instructions. The yield of RNA from each preparation was determined by UV spectrophotometry. Two microliters of messenger RNA (about 200 ng) was primed with oligo(dT) and reverse-transcribed with an avian myeloblastosis virus reverse transcriptase (cDNA cycle kit; Invitrogen). A dilution of the reverse transcription reaction was used as a template for amplification by PCR. The full-length ADAM-15 cDNA was cloned by using the following primers: sense, 5'-CGC TGT TCC GCA CTT GCT-3'; and antisense, 5'-CCG GAG AGG TCA GAG GTA GA-3'. After an initial denaturation step at 94 °C for 5 min, PCR was carried out for 35 cycles under the following conditions: denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 4 min, followed by a final cycle of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 10 min. The PCR product was electrophoresed on ethidium bromide-stained 1% (w/v) agarose gels in Tris/acetate/EDTA buffer. The PCR product had an apparent size of 2.5 kb. After gel extraction, with the QIAquick gel extraction kit (Qiagen, Valencia, CA), and its ligation into the pcDNA3.1 TOPO/V5 expression vector (Invitrogen), the PCR product was cloned. Plasmids were then purified using the Qiagen Maxiplasmid kit (Qiagen, Valencia, CA) and sequenced (Biosynthesis and Sequencing, Baltimore, MD). The pcDNA3.1 TOPO/V5 expression vector encodes the protein of interest fused with the small viral protein V5, which allows the

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detection of the exogenous expression of the protein of interest by immunoblot analysis with a mouse monoclonal anti-V5 antibody (Invitrogen).

Generation of Mutated ADAM-15 Proteins—Mutated ADAM-15 proteins were generated by PCR. The mutation of the RGD motif of ADAM-15 was performed with the following primers: forward, 5'-GCT GGC AGT GTC GTC CTA CCA GTG TGG ATT GTG ACT TG-3'; reverse, 5'-CAA GTC ACA ATC CAC ACT GGT AGG ACG ACA CTG CCA GC-3'. Once the PCR was completed, the RGD motif became mutated into an SVD sequence. The deletion of the cytoplasmic tail was also performed by PCR, by introducing a stop codon in the cytoplasmic tail coding sequence, with the following primers: forward, 5'-TCC TGG TGA TGC TTG GTG CCA GCT AGT GGT ACC GTG CC-3'; reverse, 5'-GGC ACC AAG CAT CAC CAG GAC CAA-3'. Caco2-BBE and Jurkat cells were further stably transfected with the vector alone as well as with the vector containing the cDNA encoding ADAM-15 protein or its mutants.

Transfection of Caco2-BBE Cells—Subconfluent Caco2-BBE cells were transfected with empty vector or with construct encoding ADAM-15, using TrojaneTM transfection reagent (Avanti, Alabaster, AL) according to the manufacturer's instructions, for 3–4 h in Opti-MEM I medium (Invitrogen). Transfectants were selected in culture medium containing 1.2 mg/ml G418 (Invitrogen).

Transfection of Jurkat Cells—Jurkat cells were transfected with empty vector or with construct encoding ADAM-15, by electroporation, with Multiporator[®] (Eppendorf, Hamburg, Germany), according to the manufacturer's instructions. Briefly, a cell suspension of 10^6 cells/ml in hypo-osmolar buffer (Eppendorf) was electrophoresed with 20 μ g/ml DNA using one pulse of 240 V for 40 μ s. Transfected cells were selected in culture medium containing 1 mg/ml G418 (Invitrogen).

Adhesion Assays—Jurkat cells were labeled with 5 μ M 2',7'-bis-(2-carboxyethyl)-5 (and-6) carboxyfluorescein, acetoxymethyl ester (BCECF-AM, Molecular Probes), in culture media for 30 min at 37 °C. After washing, these labeled Jurkat cells, as well as confluent Caco2-BBE cells cultured in 12-well plates, were incubated with their respective culture media with or without 10 μ g/ml mouse anti-ADAM-15 antibody, raised against the ectodomain (R&D Systems) or 10 μ g/ml mouse control antibody with the same isotype (R&D Systems), for 1 h at 37 °C. After removal of Caco2-BBE medium, Jurkat cells were added (10×10^6 cells in 400 μ l of RPMI medium per well) to confluent Caco2-BBE cells and incubated for 30 min at 37 °C. After incubation of Jurkat cells with Caco2-BBE monolayers, the media containing nonattached Jurkat cells were removed, and cells remaining in the wells were washed twice in PBS and lysed in a Triton lysis buffer (10 mM Tris, 150 mM NaCl, 3 mM EDTA, 1% Triton X-100). The fluorescence was then read using a Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Danbury, CT; excitation wavelength, 492 nm; emission wavelength, 520 nm). Experiments with transfected cells were conducted as described above, except cells were not preincubated with antibodies. Results are expressed as number of attached Jurkat cells on Caco2-BBE monolayers, based on calibration with lysates of labeled Jurkat cells, \pm S.E.

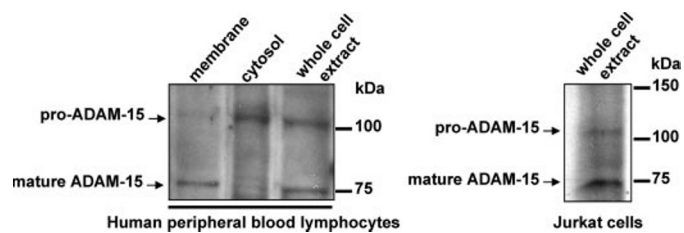


FIGURE 1. T cells express ADAM-15. Immunoblot analysis of ADAM-15 expression was performed with a rabbit anti-ADAM-15 (cytoplasmic domain) antibody, as described under "Materials and Methods," from membrane, cytosol, and whole cell extract of isolated human peripheral blood lymphocytes as well as from whole cell extract of the Jurkat T cell line. Numbers on the right indicate the molecular size of standards in kilodaltons.

Homotypic Cell Aggregation Assays—Transfected Jurkat cells were seeded in 96-well tissue culture plates at a density of 10^5 cells in 100 μ l per well and then incubated at 37 °C in 5% CO₂ and 90% humidity. Images were captured at time point 0, before incubating cells, and then at different time points, with a Nikon Eclipse TS100 microscope with a DS Camera Head DS-5M and a DS Camera Control Unit DS-L1 (Nikon, Tokyo, Japan). These images were analyzed through a digital imaging system of analysis using the ImagePro Plus[®] 5.0 software (Media Cybernetics, Silver Spring, MD). Cell aggregates were individually outlined, and the size of the delimited area was measured accounting for the relative size of aggregates. Measurements of cell aggregates from each cell suspension were exported to Excel[®] for further data analysis. A total of 71–201 cell aggregates per cell suspension were analyzed. Results are expressed as the mean of aggregates size \pm S.E.

Wound-healing Assays—Wound-healing assays were performed with the electric cell-substrate impedance sensing (ECIS, Applied BioPhysics) technology (38). The ECIS model 1600R (Applied BioPhysics) was used for these experiments. The measurement system consists of an 8-well culture dish (ECIS 8W1E plate), with the surface treated for cell culture. Each well contains a small active electrode (area = 5×10^{-4} cm²) and a large counter electrode (area = 0.15 cm²) deposited upon the bottom of each well. A lock-in amplifier, with an internal oscillator, relays to switch between the different wells, and a personal computer controls the measurement and stores the data. The entire system was obtained from Applied BioPhysics. Attachment and spreading of cells on the electrode surface change the impedance in such a way that morphological information of the attached cells can be inferred. Transfected Caco2-BBE cells and Jurkat cells were seeded together in ECIS 8W1E plates coated with 10 μ g/ml laminin I (Sigma) at a ratio of 3:1 (Caco2-BBE:Jurkat) in RPMI medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen) and 1.5 μ g/ml plasmocin (Invivogen). Control cell cultures were used, for each experiment, in which Jurkat cells were replaced by 10- μ m polystyrene microparticles (Fluka, St. Louis, MO). Once cells reached confluency, basal resistance measurements were performed for at least 1 h using the ideal frequency for Caco2-BBE cells, 500 Hz (38) and 1 V for the voltage.

For the wound-healing assays, cells were submitted to an elevated voltage pulse of 40-kHz frequency, 4.5-V amplitude, and a 30-s duration that led to death and detachment of cells

present on the small active electrode, resulting in a wound that is normally healed by cells surrounding the small active electrodes that have not been submitted to the elevated voltage pulse. Wound healing was then assessed by continuous resistance measurements.

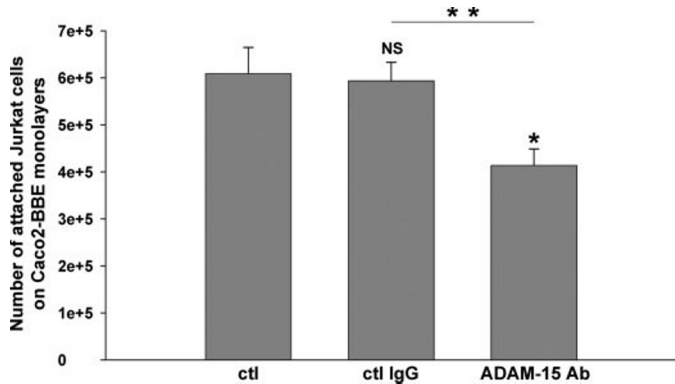


FIGURE 2. Anti-ADAM-15 antibody inhibits Jurkat cell attachment on Caco2-BBE cells. Adhesion assays were performed with Caco2-BBE cell line as an adherent monolayer and Jurkat cells as BCECF-AM fluorescent-labeled suspension cells in the presence (ADAM-15 antibody) or absence (*ctl*) of 10 μ g/ml mouse anti-ADAM-15 (ectodomain) antibody or control irrelevant antibody (*ctl IgG*). Results represent the number of attached Jurkat cells on Caco2-BBE monolayers and are expressed as the mean \pm S.E. of nine determinations. This experiment is representative of two independent experiments. *NS*, not statistically significant versus control; *, $p < 0.05$ versus control; **, $p < 0.005$; *Ab*, antibody.

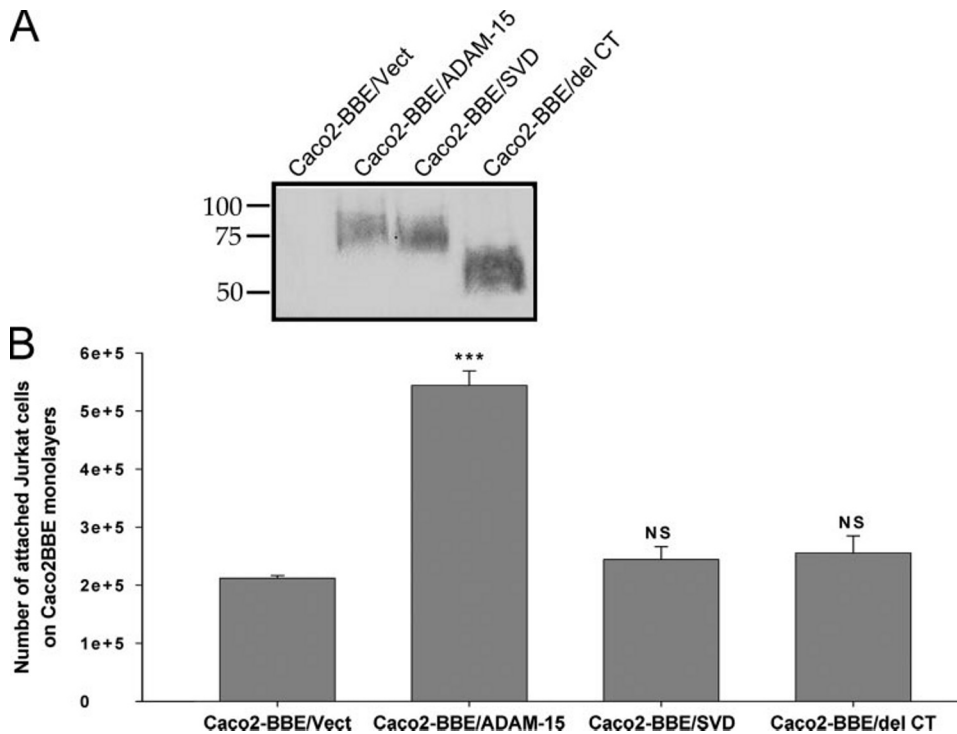


FIGURE 3. Overexpression of ADAM-15 in Caco2-BBE cells enhances Jurkat binding to Caco2-BBE cells. *A*, Western blot analysis for exogenous ADAM-15 protein expression in Caco2-BBE cells transfected with the vector alone (*Caco2-BBE/Vect*), with wild type ADAM-15 (*Caco2-BBE/ADAM-15*), or with mutated ADAM-15 proteins lacking either the RGD sequence (*Caco2-BBE/SVD*) or the cytoplasmic tail (*Caco2-BBE/del CT*) using a mouse monoclonal anti-V5 antibody. Numbers on the left indicate molecular size of standards in kilodaltons. *B*, adhesion assays were performed with Caco2-BBE/Vect, Caco2-BBE/ADAM-15, Caco2-BBE/SVD, and Caco2-BBE/del CT cells as an adherent monolayer, and wild type Jurkat cells as BCECF-AM fluorescent-labeled suspension cells. Results represent the number of attached Jurkat cells on Caco2-BBE monolayers and are expressed as the mean \pm S.E. of eight determinations. This experiment is representative of two independent experiments. *NS*, not statistically significant versus Caco2-BBE/Vect; ***, $p < 0.001$ versus Caco2-BBE/Vect.

Quantitative Reverse Transcription-PCR Analysis—T cell aggregation assays were performed as described above. After 6 h of incubation, total RNA was extracted from cells using TRIzol reagent (Invitrogen), reverse-transcribed using the Superscript III first-strand system (Invitrogen), and TNF α cDNA was amplified using the iQ SYBR Green Supermix (Bio-Rad) and the real time iCycler sequence detection system (Bio-Rad). TNF α cDNA was amplified at 95 $^{\circ}$ C for 3 min followed by 45 cycles of 95 $^{\circ}$ C for 30 s and 60 $^{\circ}$ C for 1 min with 10 μ M gene-specific primers. GAPDH expression levels were used as a reference. The primers used are as follow: TNF α forward, 5'-AGC CCA TGT TGT AGC AAA CC-3', and TNF α reverse, 5'-TGA GGT ACA GGC CCT CTG AT-3'; GAPDH forward, 5'-GTC GGA GTC AAC GGA TTT GG-3', and GAPDH reverse, 5'-AAG CTT CCC GTT CTC AGC CT-3'. For graphical representation of quantitative PCR data, raw threshold cycle (C_t) values obtained for each transfectant were deducted from the C_t values obtained for internal GAPDH transcript levels using the $\Delta\Delta C_t$ method as follows: $\Delta\Delta C_t = (C_{t,TNF\alpha} - C_{t,GAPDH})_{transfectant} - (C_{t,transfectant} - C_{t,vector})_{TNF\alpha}$ and final data were derived from $2^{-\Delta\Delta C_t}$ (44). Results represent the data obtained from two independent experiments and are expressed as the relative content of TNF α mRNA present in each transfectant \pm S.E.

Statistical Data—Student's *t* test was used to determine statistical significance of the data obtained in all assays.

RESULTS

The Binding of Jurkat Cells to Caco2-BBE Cells Is Inhibited by an Anti-ADAM-15 Antibody Raised against the Ectodomain—We have previously shown that ADAM-15 is expressed in human normal intestine and in the intestinal epithelial cell line Caco2-BBE (38). Because ADAM-15 has been shown to bind to $\alpha v\beta 3$ and $\alpha 5\beta 1$ in an RGD-dependent manner on hematopoietic cells (33), we assessed the role of ADAM-15 in cell-cell interactions of IEC/T lymphocytes. For this purpose, we performed adhesion assays of Jurkat cells, labeled with the fluorescent dye BCECF on confluent Caco2-BBE cells, as described under "Materials and Methods." Because human peripheral blood lymphocytes as well as human hematopoietic cell lines, including Jurkat, express ADAM-15 (Fig. 1) (31, 36, 37), both Caco2-BBE and Jurkat cell lines were preincubated with or without 10 μ g/ml of mouse anti-ADAM-15 antibody raised against ADAM-15 ectodomain or with a control irrelevant antibody. Labeled Jurkat cells were added to confluent

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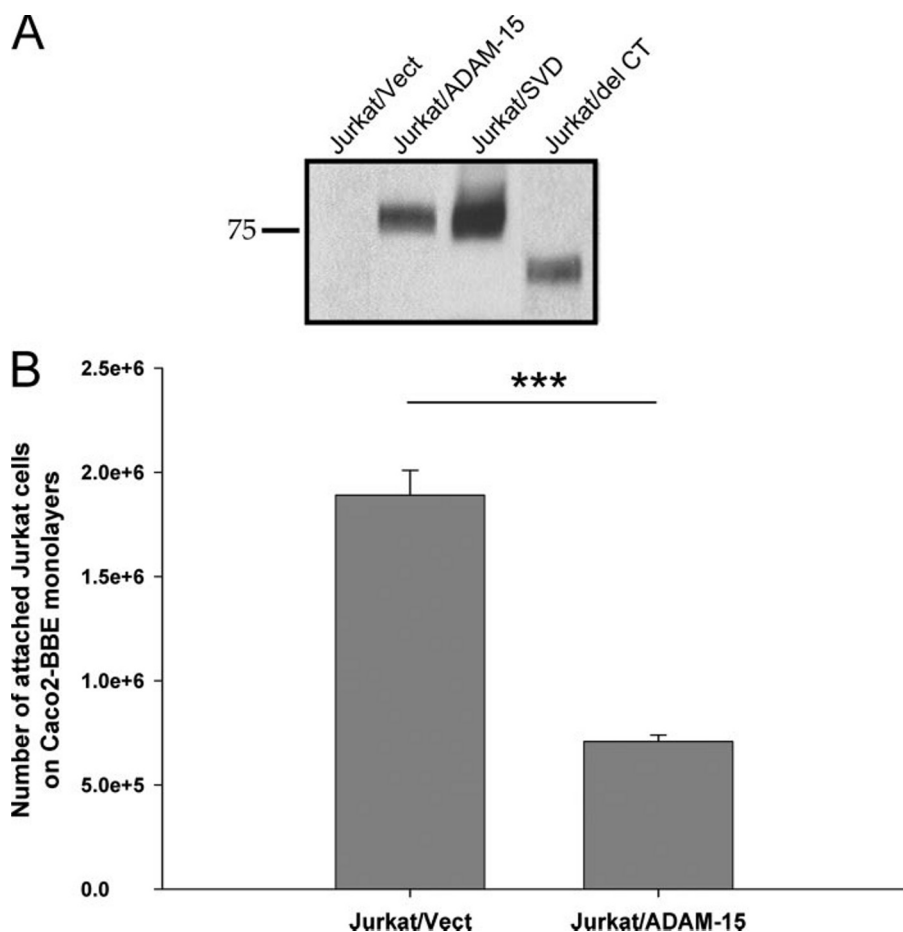


FIGURE 4. Overexpression of ADAM-15 in Jurkat cells inhibits Jurkat binding to Caco2-BBE cells. *A*, Western blot analysis for exogenous ADAM-15 protein expression in Jurkat cells transfected with the vector alone (*Jurkat/Vect*), with wild type ADAM-15 (*Jurkat/ADAM-15*), or with mutated ADAM-15 proteins lacking either the RGD sequence (*Jurkat/SVD*), or the cytoplasmic tail (*Jurkat/del CT*), using a mouse monoclonal anti-V5 antibody. Numbers on the left represent molecular size of standard in kilodaltons. *B*, adhesion assays were performed with wild type Caco2-BBE cells as an adherent monolayer and Jurkat/Vect or Jurkat/ADAM-15 cells as BCECF-AM fluorescent-labeled suspension cells. Results represent the number of attached Jurkat cells on Caco2-BBE monolayers and are expressed as the mean ± S.E. of 12 determinations. This experiment is representative of two independent experiments. ***, $p < 0.001$.

Caco2-BBE cells, and binding of Jurkat cells to Caco2-BBE monolayers was determined by reading the fluorescence after lysis of adherent cells. Fig. 2 shows that anti-ADAM-15 antibody was significantly inhibited by ~30% Jurkat cell binding to Caco2-BBE cells ($413,750 \pm 34,855$ attached cells *versus* $608,775 \pm 55,760$ without antibody). In contrast, the control antibody did not have any significant effect on Jurkat cell attachment ($593,590 \pm 39,275$ attached cells *versus* $608,775 \pm 55,760$ without antibody). These results suggest that ADAM-15 is involved in IEC/T lymphocyte interactions.

Overexpression of ADAM-15 in Caco2-BBE Cells Enhances the Binding of Jurkat Cells—To further determine whether epithelial cell-derived and/or T cell-derived ADAM-15 is involved in IEC/T lymphocyte cell-cell interactions, we performed cell adhesion assays using either Caco2-BBE overexpressing ADAM-15 and wild type Jurkat cells or wild type Caco2-BBE cells and Jurkat cells overexpressing ADAM-15.

Caco2-BBE cells were transfected with the pcDNA3.1 TOPO/V5 expression vector alone (Caco2-BBE/Vect) or containing the cDNA encoding ADAM-15 protein (GenBank™

accession number AY518542, see Ref. 38) (Caco2-BBE/ADAM-15) fused with the viral V5 protein. Western blot analysis, performed on whole cell lysates from transfected cells with anti-V5 antibody showed that exogenous ADAM-15 was expressed in Caco2-BBE cells (Fig. 3A). Binding assays of BCECF-labeled Jurkat cells were then performed on confluent Caco2-BBE/Vect and Caco2-BBE/ADAM-15 cells. Fig. 3B shows that overexpression of ADAM-15 in Caco2-BBE cells dramatically enhanced Jurkat cells binding with Caco2-BBE monolayers ($543,621 \pm 25,161$ attached cells on Caco2-BBE/ADAM-15 monolayers *versus* $211,732 \pm 4725$ attached cells on Caco2-BBE/Vect monolayers). This suggests that ADAM-15 molecules present on Caco2-BBE cells are involved in the binding with Jurkat cells.

Overexpression of ADAM-15 in Jurkat Cells Enhances Their Aggregation—To determine whether ADAM-15 present on Jurkat cells is also involved in the binding with Caco2-BBE cells, Jurkat cells were also transfected with ADAM-15 (GenBank™ accession number AY518542, see Ref. 38) (*Jurkat/ADAM-15*) or with the vector alone (*Jurkat/Vect*). Western blot analysis, performed on transfected cell lysates, with anti-V5 antibody,

showed that exogenous ADAM-15 was expressed in Jurkat cells (Fig. 4A). Binding assays of BCECF-labeled Jurkat/Vect or BCECF-labeled Jurkat/ADAM-15 cells were then performed on confluent wild type Caco2-BBE cells. Interestingly, overexpression of ADAM-15 at the cell surface membrane of Jurkat cells drastically decreased the binding of Jurkat cells with Caco2-BBE cells by ~60% (Fig. 4B; $708,570 \pm 30,225$ attached Jurkat/ADAM-15 cells *versus* $1,890,225 \pm 118,550$ attached Jurkat/Vect cells).

We next examined the underlying reason for decreased adhesion of Jurkat/ADAM-15 cells to wild type Caco2-BBE cells. We observed that Jurkat/ADAM-15 cells formed much bigger aggregates than did Jurkat/Vect cells (Fig. 5A). Therefore, to further determine the role of ADAM-15 in the formation of these clusters, we performed homotypic cell adhesion assays. Jurkat/Vect and Jurkat/ADAM-15 cells were seeded as a monocellular suspension at the same density in 96-well plates and incubated at 37 °C, 5% CO₂. Pictures were then taken at different time points. Fig. 5B represents the pictures of cell suspensions taken at different time points after seeding, and Fig.

5C represents the size of cell aggregates that formed each cell suspension over time. Fig. 5B shows that, at time point 0 (T_0), both Jurkat/Vect and Jurkat/ADAM-15 cells were seeded as a

monocellular suspension. Then both transfectants started to form aggregates of increasing size over time (Fig. 5, B and C). However, the clusters formed by Jurkat/ADAM-15 cells were

much larger than the ones formed by Jurkat/Vect cells (656.4 ± 21.2 for Jurkat/Vect cells *versus* 993.6 ± 47.8 for Jurkat/ADAM-15 cells at 30 min, and 1260.7 ± 72.8 for Jurkat/Vect cells *versus* 3616.7 ± 342.3 for Jurkat/ADAM-15 cells at 5 h; see Fig. 5C). These findings suggest that ADAM-15 plays a role in T lymphocyte aggregation.

These data also provide an explanation for the decreased number of Jurkat/ADAM-15 cells that attached to Caco2-BBE monolayers in comparison with the number of attached Jurkat/Vect cells, during heterotypic cell binding experiments (Fig. 4B). Indeed, Jurkat/ADAM-15 cells are probably more likely to bind to each other instead of binding to Caco2-BBE cells.

Both ADAM-15 RGD Sequence and Cytoplasmic Tail Are Involved in Heterotypic Cell-Cell Interactions between Caco2-BBE Monolayers and Jurkat Cells—To determine the molecular mechanisms underlying ADAM-15-mediated cell-cell interactions between IEC and T lymphocytes, we transfected Caco2-BBE cells with constructs encoding mutated ADAM-15 proteins. Mutated ADAM-15 proteins either lacking the RGD motif (Fig. 6, SVD) or the cytoplasmic tail (Fig. 6, del CT) were generated by reverse transcription-PCR as described under “Materials and Methods.” After stable transfection of Caco2-BBE cells with these constructs encoding mutated ADAM-15 proteins (Caco2-BBE/SVD and Caco2-BBE/del CT), we confirmed, by Western blot, that these mutated ADAM-15 proteins were expressed (Fig. 3A). We then performed heterotypic cell adhesion assays using transfected Caco2-BBE monolayers and wild type Jurkat cells. As represented in Fig. 3B, both the mutation of the RGD sequence (Caco2-BBE/SVD) and the deletion of the cytoplasmic tail (Caco2-BBE/del CT) abolished ADAM-15-mediated enhancement of Jurkat cell adhesion to

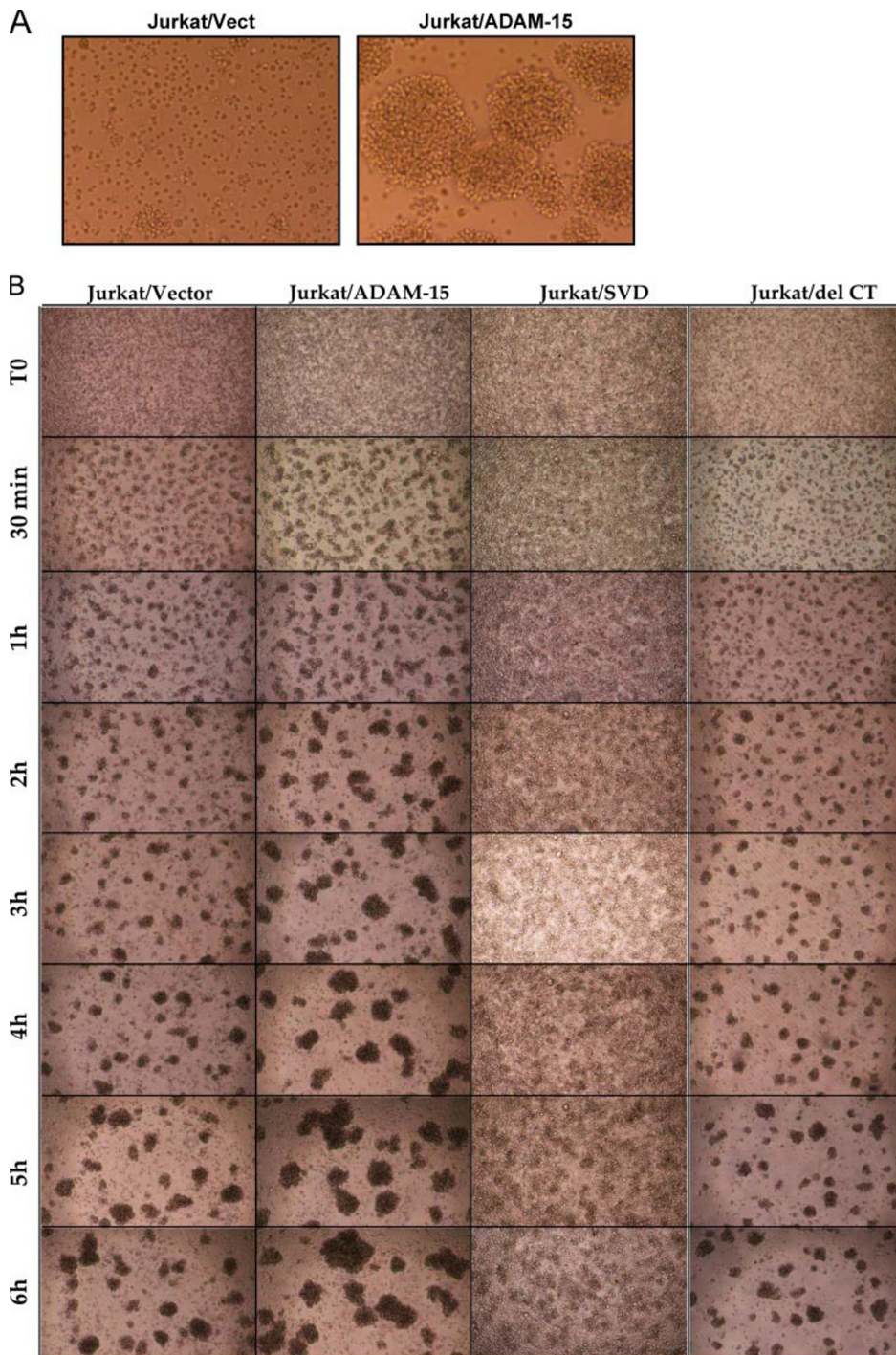


FIGURE 5. Overexpression of ADAM-15 in Jurkat cells enhances homotypic binding. A, pictures representing the ability of Jurkat cells transfected with ADAM-15 (*Jurkat/ADAM-15*) to form aggregates compared with Jurkat cells transfected with the vector alone (*Jurkat/Vect*) during routine cell culture. B, aggregation assays were performed as described under “Materials and Methods” using Jurkat/Vect and Jurkat/ADAM-15 cells, as well as Jurkat cells transfected with mutated ADAM-15 proteins lacking either the RGD sequence (*Jurkat/SVD*) or the cytoplasmic tail (*Jurkat/del CT*). Pictures were taken at different time points after initial seeding as a monocellular suspension (T_0). This experiment is representative of three independent experiments. Each picture represents 1 well out of 3 wells per condition. C, cell aggregate size was analyzed through a digital imaging system of analysis using the ImagePro Plus® 5.0 software as described under “Materials and Methods.” A total of 71–201 cell aggregates per cell suspension were analyzed. Results are expressed as the mean of aggregates size \pm S.E. **, $p < 0.005$, and ***, $p < 0.001$ *versus* Jurkat/Vect for each incubation time.

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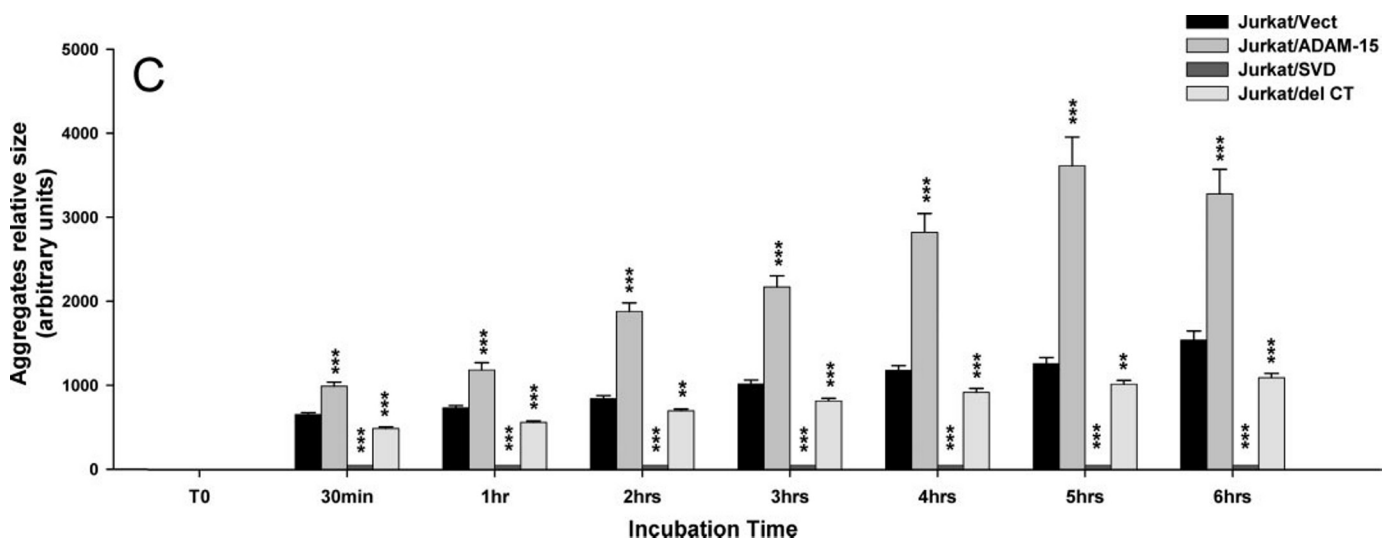


FIGURE 5—continued

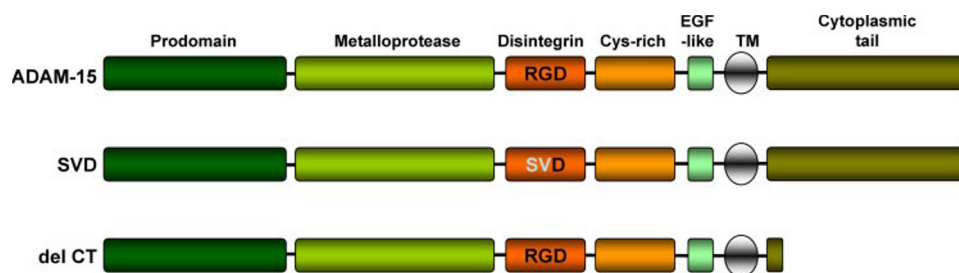


FIGURE 6. **Schematic representation of the mutations generated from wild type ADAM-15 protein.** Mutations of wild type ADAM-15 were generated by site-directed mutagenesis as described under "Materials and Methods." The RGD motif was mutated and replaced by an SVD sequence, and the cytoplasmic domain was deleted by introducing a stop codon in the cytoplasmic tail coding sequence (*del CT*).

Caco2-BBE monolayers (Caco2-BBE/ADAM-15). This suggests that both the RGD sequence and the cytoplasmic domain are required for these ADAM-15-mediated cell-cell interactions.

Both ADAM-15 RGD Sequence and Cytoplasmic Tail Are Involved in ADAM-15-mediated T Cell Aggregation—We then aimed at determining the molecular mechanisms of ADAM-15-mediated T cell aggregation. Jurkat cells were stably transfected with the abovementioned constructs encoding mutated ADAM-15 proteins (Fig. 6), and the different transfectants were assessed for mutated ADAM-15 protein expression (Fig. 4A). Homotypic cell-cell interaction assays were performed using these transfected Jurkat cells as described under "Materials and Methods." We found that Jurkat cells overexpressing ADAM-15 with a mutated RGD integrin binding sequence (Jurkat/SVD) tended to form groups but did not form any aggregates as seen for the other transfected Jurkat cells (Fig. 5, B and C). The deletion of the cytoplasmic tail (Jurkat/del CT) abolished ADAM-15-mediated enhancement of Jurkat cell aggregation (Jurkat/ADAM-15) (aggregates size, 489.8 ± 19.3 for Jurkat/del CT cells versus 993.6 ± 47.8 for Jurkat/ADAM-15 cells at 30 min, and 1017 ± 45.7 for Jurkat/del CT cells versus 3616.7 ± 342.3 for Jurkat/ADAM-15 cells at 5 h; see Fig. 5, B and C). These results indicate that both the RGD motif and the cytoplasmic tail are involved in T cell homotypic interactions

and that the RGD sequence is fundamental for the establishment of such cell-cell interactions.

Epithelial ADAM-15-mediated Increase of Cell-Cell Interactions between Caco2-BBE and Jurkat Cells Is Associated with Enhanced Epithelial Restitution—During intestinal inflammation, intestinal epithelium undergoes damages followed by epithelium repair also called epithelial restitution. We therefore investigated whether ADAM-15-mediated cell-cell inter-

actions between Caco2-BBE and Jurkat cells could affect the processes of epithelium wound repair. Transfected Caco2-BBE cells were co-seeded and co-cultured with Jurkat cells or inert 10- μ m polystyrene beads in ECIS 8W1E plates coated with 10 μ g/ml of laminin I as described under "Materials and Methods." After cells reached confluency, basal resistance measurements were performed for at least 1 h, and cells were submitted to an elevated voltage pulse leading to a wound. As represented in Fig. 7, cells exhibited a stable basal resistance that drastically dropped upon application of the high voltage pulse (arrow), indicating the formation of the wound. Fig. 7A shows that for Caco2-BBE/Vect cells co-cultured with polystyrene beads (dotted line), wound-healing is achieved within 10 h following the wound. However, in accordance with our previous findings (38), overexpression of ADAM-15 in Caco2-BBE cells greatly inhibited the mechanisms of wound repair when co-cultured with inert beads (Fig. 7B, dotted line). Interestingly, we found that upon mutation of ADAM-15 RGD motif or deletion of the cytoplasmic tail, cells that were co-seeded with beads (Fig. 7, C and D, respectively; dotted line) were able to recover from the injury in the same time frame as Caco2-BBE/Vect cells (Fig. 7A, dotted line). These findings suggest that both the RGD sequence and the cytoplasmic tail are involved in ADAM-15-mediated inhibition of wound healing (38).

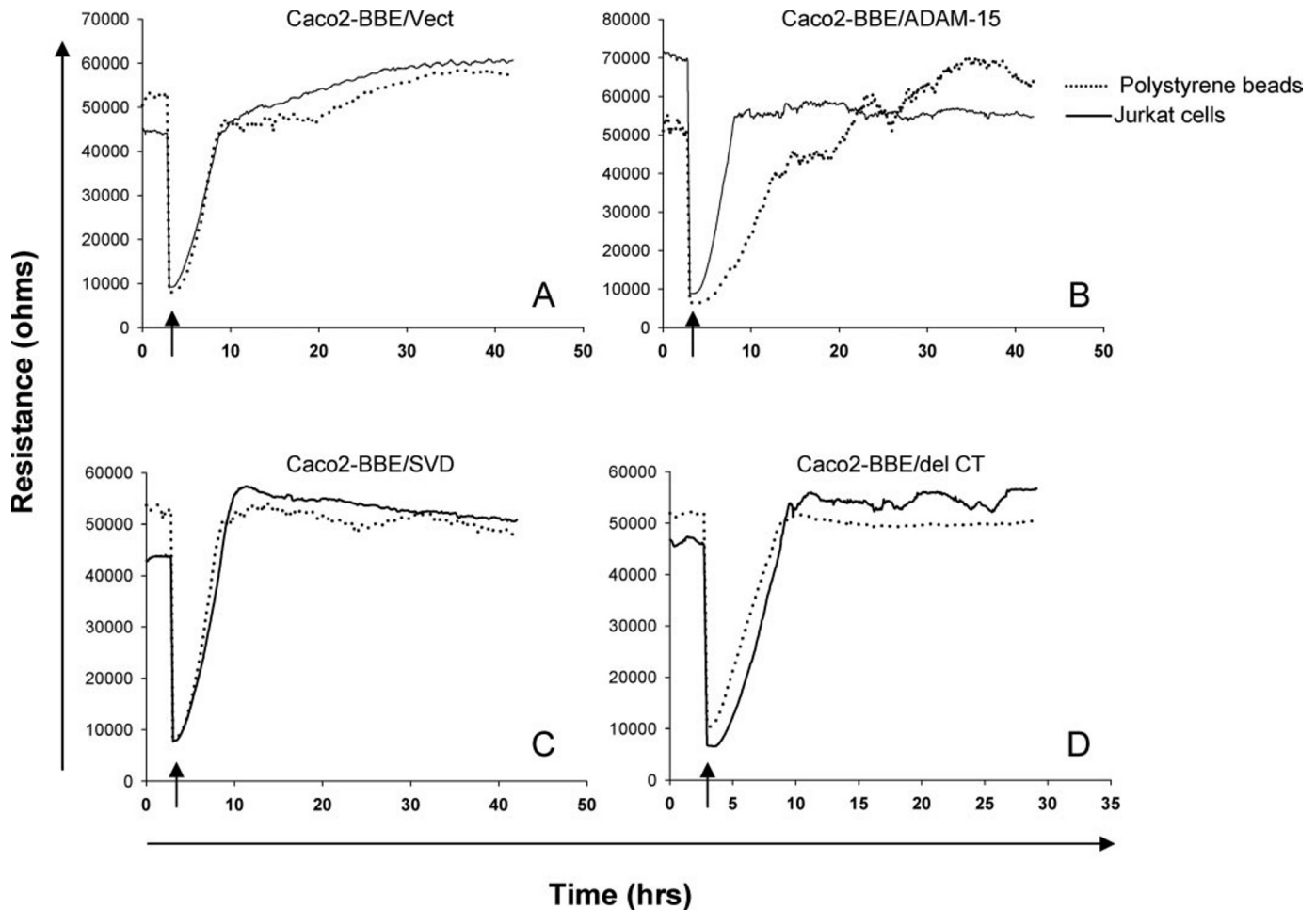


FIGURE 7. **ADAM-15-mediated T cell adhesion to IEC is associated with enhanced wound healing.** Caco2-BBE/Vect (A), Caco2-BBE/ADAM-15 (B), Caco2-BBE/SVD (C), and Caco2-BBE/del CT cells (D) were co-seeded with Jurkat cells (solid line) or with inert polystyrene beads used as a control (dotted line), as described under "Materials and Methods." Confluent co-cultures were wounded (arrow) through application of a high voltage pulse of 40-kHz frequency, 4.5-V amplitude, and a 30-s duration. The wound was then allowed to heal, and the level of recovery was assessed by continuous resistance measurements.

Fig. 7 also shows that when Caco2-BBE/Vect, Caco2-BBE/SVD, and Caco2-BBE/del CT cells were co-cultured with Jurkat cells, the recovery was not affected by the presence of Jurkat cells (Fig. 7, A, C, and D, respectively; solid line). However, for Caco2-BBE/ADAM-15 cells, the presence of Jurkat cells increased the processes of cell recovery (Fig. 7B, solid line) in comparison with Caco2-BBE/ADAM-15 cells cultured in the presence of beads (Fig. 7B, dotted line) and also in comparison with Caco2-BBE/Vect, Caco2-BBE/SVD, and Caco2-BBE/del CT cells co-cultured with Jurkat cells (Fig. 7, A, C, and D, respectively; solid line). Interestingly, these last findings strongly correlate with the level of Jurkat cell adhesion on the different transfected Caco2-BBE cells (Fig. 3B). This suggests that epithelial ADAM-15-mediated cell adhesion of T lymphocytes to IEC increases wound-healing mechanisms.

ADAM-15-mediated Aggregation of T Cells Is Associated with an Increase of TNF α mRNA Expression—Because lymphoid aggregates are observed during intestinal inflammation (12–16), we assessed the consequences of ADAM-15-mediated aggregation of T lymphocytes on TNF α expression, which is a cytokine that plays a major role in inflammation (16). T cell homotypic adhesion assays were performed as described above using the different transfectants of Jurkat cells, and after 6 h of

incubation, cells were harvested and assessed for TNF α mRNA expression by real time PCR. Fig. 8 shows that upon overexpression of ADAM-15 (ADAM-15), the quantity of TNF α mRNA present in those cells tends to increase in comparison with cells transfected with the vector alone (Vect). However, for cells transfected with ADAM-15 lacking the RGD motif (SVD) or the cytoplasmic tail (del CT), the expression of TNF α mRNA was drastically decreased (Fig. 8), which indicates the requirement for both the RGD motif and the cytoplasmic tail of ADAM-15.

DISCUSSION

The purpose of this study was to investigate the role of ADAM-15 in heterotypic cell-cell interactions between IEC and T lymphocytes. Cell-cell interactions play an important role in many different aspects of immunobiology. These interactions are involved in cell localization, effector recognition, and activation phenomena. The process of lymphocyte recruitment from the circulation into the mucosa, also termed "homing," requires the extravasation of these leukocytes from the microvasculature and their subsequent migration into the epithelial compartment. Cell-cell interactions, representing a key step in this lymphocyte trafficking,

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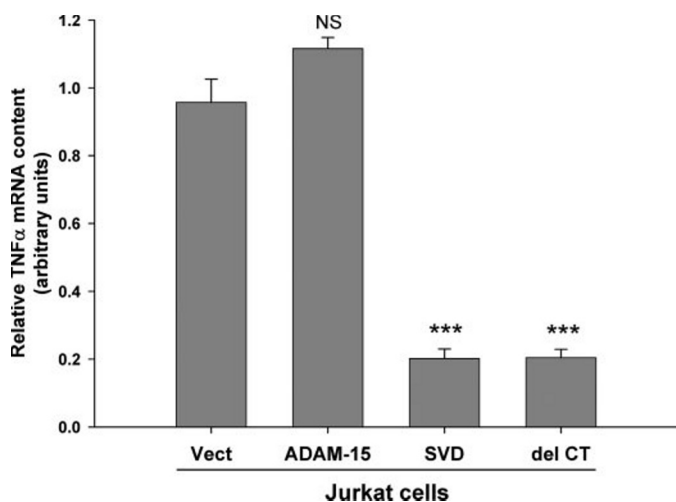


FIGURE 8. ADAM-15-mediated T cell aggregates are associated with TNF α mRNA expression. TNF α transcripts present in transfected Jurkat cells were quantified by real time PCR after cell aggregation assays (6 h incubation) as described under "Materials and Methods." For graphical representation of quantitative PCR data, raw threshold cycle (C_t) values obtained for each transfectant were deducted from the C_t values obtained for internal GAPDH transcript levels using the $\Delta\Delta C_t$ method as follows: $\Delta\Delta C_t = (C_{t,TNF\alpha} - C_{t,GAPDH})_{transfectant} - (C_{t,transfectant} - C_{t,vector})_{TNF\alpha}$, and final data were derived from $2^{-\Delta\Delta C_t}$ (44). Results represent the data obtained from two independent experiments and are expressed as the relative content of TNF α mRNA present in each transfectant \pm S.E. NS, not statistically significant; ***, $p < 0.001$ versus Vect.

involve adhesion molecules present on endothelial/epithelial cells as well as on lymphocytes.

We previously reported that ADAM-15 is expressed in IEC (38). Human peripheral blood lymphocytes as well as human hematopoietic cell lines, including Jurkat, have been shown to express ADAM-15 (31, 36, 37; and this study). Interestingly, ADAM-15 contains an RGD integrin-binding motif in its disintegrin domain (31) that has been shown to mediate the binding of ADAM-15 to $\alpha v\beta 3$ and $\alpha 5\beta 1$ on hematopoietic cells (33).

Our results show for the first time that ADAM-15 is involved in heterotypic cell-cell interactions between IEC and T lymphocytes. First, Jurkat cell attachment to Caco2-BBE monolayers was inhibited by an anti-ADAM-15 antibody raised against the ectodomain of ADAM-15. Second, ADAM-15 overexpression in Caco2-BBE cells enhanced Jurkat cell adhesion to Caco2-BBE monolayers. These results suggest that epithelial ADAM-15 binds to ligand(s) that is/are expressed on the cell surface of T lymphocytes. Our finding that overexpression of ADAM-15 with a mutation in the RGD motif in Caco2-BBE monolayers decreases Jurkat cell adhesion shows that ADAM-15-mediated binding of T cells on IEC is dependent on the RGD sequence. This finding points to $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins as the potential epithelial ADAM-15 binding partners expressed on T lymphocyte membranes since *in vitro* experiments have shown that the disintegrin domain of ADAM-15 interacts with $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins present on hematopoietic cells (33) and that ADAM-15 interacts with these integrins in a RGD-dependent manner (32, 33). However, the findings that $\alpha 5\beta 1$, $\alpha 9\beta 1$, and αv integrins are faintly or not expressed in the normal human intestinal epithelium (46–49) are not in favor of an involvement of T cell-derived ADAM-15 in cell-cell interactions between IEC and T lymphocytes.

Heterotypic cell adhesion assays using Jurkat cells transfected with ADAM-15 did not allow us to further determine the role of ADAM-15 present on T cells in heterotypic cell-cell interactions with IEC. However, these experiments pointed to another aspect of ADAM-15 involvement in cell-cell interactions and raised ADAM-15 as another molecule involved in aggregation of T cells. This finding provides an explanation for the results obtained in the heterotypic cell adhesion assays using wild type Caco2-BBE cells and Jurkat cells overexpressing ADAM-15; and this finding is in agreement with a previous report showing that overexpression of ADAM-15 in the fibroblastic cell line NIH3T3 enhances homotypic cell-cell interactions (35). This can be explained by the fact that lymphocytes express both ADAM-15 and at least two of its ligands, *i.e.* $\alpha v\beta 3$ and $\alpha 5\beta 1$ (31, 32, 34, 36, 37). Moreover, our finding that Jurkat cell aggregation depends on the RGD motif suggests that these integrins are ADAM-15 binding partners involved in these cell-cell interactions.

By promoting T cell aggregation, ADAM-15 may have a role in establishing or supporting the interactions that promote the formation of an immunological synapse or microdomain containing appropriate signaling proteins that become important for the initiation of signaling events involved in T cell activation. This latter speculation is supported by the finding that the cytoplasmic tail of ADAM-15 induces signaling transduction in immune cells (37). In addition, it is known that integrins are also able to transduce signals in T cells (50).

Our results show that ADAM-15 cytoplasmic tail is also required for the establishment of both heterotypic and homotypic cell-cell interactions. We indeed found that Jurkat cell attachment to Caco2-BBE monolayers was decreased when Caco2-BBE cells overexpressed ADAM-15 proteins with a deleted cytoplasmic tail in comparison with the attachment to Caco2-BBE monolayers overexpressing wild type ADAM-15 proteins. Similarly the deletion of the ADAM-15 cytoplasmic tail abolished exogenous ADAM-15-induced enhancement of Jurkat cell aggregation. These results strongly suggest that not only the ADAM-15 ectodomain but also the cytoplasmic domain play a role in cell-cell interactions, possibly through signaling transduction that helps maintain or enhance cell-cell interactions. The difference observed in Jurkat cell aggregation between cells overexpressing ADAM-15 with a mutated RGD sequence or with a deleted cytoplasmic tail suggests that ADAM-15 binding to integrins through the RGD sequence is the primary event for these cell-cell interactions. Our observation that Jurkat cells overexpressing ADAM-15 with a deleted cytoplasmic tail formed smaller aggregates than Jurkat cells transfected with the vector alone suggests that in the absence of cytoplasmic tail, the events that initiate the formation of T cell aggregates are impaired or delayed.

The physiological relevance of these findings lies in the fact that lymphoid aggregates are present during intestinal inflammation (12–16) and in our finding that ADAM-15-mediated T cell aggregation was associated with increased expression of TNF α , which is an important cytokine involved in intestinal inflammation (16). Upon mutation of the RGD domain or deletion of the cytoplasmic tail, when cell aggregation was abolished or reduced, respectively, the expression of TNF α mRNA was

greatly decreased. Upon overexpression of ADAM-15, the increase of TNF α mRNA expression was not statistically significant despite these cells forming much bigger aggregates than cells transfected with the vector alone. This could be due to the fact that in our experimental conditions the presence of endogenous ADAM-15 in cells is sufficient to lead to a maximal amount of TNF α mRNA expression. However, the decrease of TNF α mRNA levels found in cells transfected with mutated ADAM-15 proteins shows that ADAM-15 is involved in the increase of TNF α mRNA expression. Moreover, we found that mutation of the RGD motif and deletion of the cytoplasmic tail led to an equivalent decrease of TNF α mRNA expression, whereas mutation of the RGD domain abolished the formation of cell aggregates, and deletion of the cytoplasmic tail only reduced cell aggregation. This suggests that the increase of TNF α mRNA expression does not only depend on cell aggregation but might also depend on ADAM-15-mediated cell signaling. Further studies are needed to determine the mechanisms of ADAM-15-mediated increase of TNF α expression in T cells.

Our work shows that ADAM-15 is able to mediate heterotypic cell-cell interactions between human T lymphocytes and human IEC, as well as human T lymphocyte homotypic cell-cell interactions. Some other proteins have been described to mediate homo- and heterotypic cell-cell interactions. For example, N-cadherin has been shown to be involved in T cell aggregation (28) as well as in heterotypic adhesion of malignant T cells to epithelia (27). LFA-1 is also able to mediate phorbol ester-induced homotypic adhesion of leukocytes (51) and interferon γ -induced homotypic adhesion of monocytes (52), as well as heterotypic cell-cell interactions between T lymphocytes and endothelial cells (53).

Adhesive interactions of leukocytes are very important in the development of immune functions such as leukocyte homing or cytotoxic T lymphocyte-mediated killing (54, 55). It is interesting to speculate that ADAM-15-mediated cell adhesion might be involved in such processes. Our recent observation (39) of ADAM-15 up-regulation during IBD and the spatial relationships of ADAM-15 and its binding partners already suggested an involvement of ADAM-15 in leukocyte transepithelial and transendothelial migration that constitute a hallmark of IBD. In this study we further demonstrate that ADAM-15 plays a role in T cell adhesion to IEC. In addition, our wound-healing experiments on transfected Caco2-BBE cells co-cultured with Jurkat cells showed that Jurkat cells enhance wound repair mechanisms and that this phenomenon involves ADAM-15. The strong correlation between the levels of Jurkat cell attachment on the different Caco2-BBE cell transfectants and the effects of Jurkat cells on wound healing strongly suggest that epithelial ADAM-15-mediated cell adhesion of T lymphocytes to IEC promotes mechanisms of epithelium repair. These findings are of great physiological significance because intestinal inflammation is characterized by disruption of the intestinal epithelium, which is followed by epithelial restitution. The mechanisms of T lymphocyte-induced increase of wound healing through ADAM-15 remain to be elucidated. However, we can speculate that this could be due to the fact that T lymphocytes compete with neighboring IEC for interactions with ADAM-15, which

could lead to decreased homotypic cell-cell interactions between IEC and therefore increased motility of IEC. It is also possible that T cells become activated upon adhesion to IEC through ADAM-15 and produce factors that increase IEC wound healing. Such factors have already been described and include interleukin-2 (56) as well as interleukin-22, which is increased in active Crohn disease (45).

Further studies are needed to elucidate the molecular mechanisms underlying ADAM-15-mediated homotypic T cell adhesion and heterotypic cell-cell interactions of T cells with IEC. The mechanisms of TNF α expression and epithelial restitution subsequent to these interactions, respectively, remain to be determined as well.

Altogether these results raise ADAM-15 as a very important molecule involved in both homotypic and heterotypic cell-cell interactions and point to ADAM-15 as a mediator of T lymphocyte transepithelial migration during intestinal inflammation. These data also suggest ADAM-15 as a possible target in the prevention of inappropriate T cell activation resulting in some pathologies.

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