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Adenovirus inhibition by peptidomimetic integrin antagonists

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Abstract

Many viruses and bacterial pathogens are capable of exploiting host cell surface integrins during their replication cycles. The ligands for many integrins contain an arginine–glycine–aspartic acid (RGD) amino acid sequence that is essential for protein–protein interaction. Human adenovirus particles contain this sequence in the penton base protein, and previous studies support a role for this RGD in integrin-dependent internalization of the virus by the cell. As synthetic peptidomimetics of RGD have been shown in other experimental systems to be antagonists of the activities of specific integrins both in vitro and in vivo, we sought to determine whether these small molecules are antagonists of adenovirus infection. Such compounds inhibited viral infection of cultured cells with similar rank order potency to that determined in assays utilizing purified extracellular matrix proteins as integrin ligands. The maximal level of inhibition achieved with the peptidomimetics was comparable to that of RGD-containing peptides, whereas no significant effects were apparent with an RGE-containing peptide. An engineered adenovirus having a mutated RGD sequence in the penton base was not susceptible to the inhibition. The results obtained with these synthetic antagonists, which have varied structures and potencies, suggest that integrins interact with adenoviral RGD in a manner similar to that of other protein ligands such as vitronectin. Furthermore, the results confirm the role of RGD in the replication cycle, and suggest peptidomimetic compounds may be useful antimicrobial agents in the treatment of a variety of diseases.

Keywords: Adhesion; RGD; Integrin; Adenovirus; Antiviral

1. Introduction

The integrins are a family of large alpha/beta heterodimeric membrane proteins that function as cell adhesion and signal transducing molecules affecting proliferation, survival, differentiation, and migration (Ruoslahti, 1991; Hynes, 1992). Integrins are expressed on most cell types al-

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though the individual family members are often restricted to various tissues, or are induced only under certain conditions. The specific combination of subunits forming each integrin determines the selective recognition of a variety of soluble and insoluble ligands. Binding by many integrins including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$, and $\alpha_5\beta_1$ has been shown to be dependent upon the presence in the ligand of the amino acid sequence arginine–glycine–aspartate (RGD), but is also influenced by other sequences at adjacent and non-adjacent sites (Koivunen et al., 1995; Mould et al., 1998; Kraft et al., 1999; Redick et al., 2000).

The binding and internalization of pathogens by host cells are critical steps in the development of many infectious diseases. This process is facilitated by the presence of specific protein structures on the invading microorganism that mirror those present on normal host proteins. Microorganisms have evolved to exploit this similarity to promote interaction with specific cell surface receptors and subsequent tissue colonization. Several studies have implicated a role for integrins in the binding or uptake of viral, bacterial, and protozoan pathogens by cells (Isberg and Tran Van Nhieu, 1994; Kerr, 1999). These pathogens include several types of adenovirus (Wickham et al., 1993; Mathias et al., 1998), foot and mouth disease virus (Jackson et al., 1997; Neff et al., 1998), coxsackievirus A9, *Borrelia burgdorferi*, *Pneumocystis carinii*, *Yersinia*, *Streptococcus*, *Plasmodium falciparum*, *Neissera*, hantaviruses, adeno-associated virus, and HIV (Kerr, 1999).

One of the most extensively characterized pathogens with respect to integrin interactions is adenovirus (Nemerow and Stewart, 1999). Adenovirus is a significant cause of respiratory and gastrointestinal infections in children (Brandt et al., 1969; Wadell et al., 1987), and has also been widely used as a vector to deliver genes into a variety of tissues. There are over 40 different serotypes of human adenovirus and binding specificities with cellular receptors determine tissue tropism. Infection of cells by adenovirus is a multistep process. The viruses appear to initially interact with several types of receptors on the cell surface (Stevenson et al., 1995; Bergelson et al., 1997; Hong et al., 1997; Tomko et al., 1997; Arnberg et al., 2000) via the viral fiber protein. Either simultaneously or subsequently, RGD sequences in the penton base protein mediate interactions with cellular integrins (Stewart et al., 1997). This interaction is required to promote efficient internalization of the virus particle. Infection of cultured human cells has been shown to be inhibited by RGD-containing peptides, by antibodies that block the function of $\alpha_v\beta_3$ or $\alpha_v\beta_5$, and by purified soluble $\alpha_v\beta_5$ (Belin and Boulanger, 1993; Wickham et al., 1993; Mathias et al., 1998). In addition, $\alpha_v\beta_5$ appears to promote permeablization of the endocytic vesicle after internalization (Wickham et al., 1994). Recombinant viruses in which the RGD sequence has been mutated exhibit delayed replication (Bai et al., 1993). Furthermore, adenovirus-mediated gene delivery is stimulated by up-regulation of $\alpha_v\beta_3$ or $\alpha_v\beta_5$ in cells with normally low levels of these integrins (Huang et al., 1995; Davison et al., 1997; Hashimoto et al., 1997), or by transfection of $\alpha_v\beta_5$ into low-expressing cells (Wickham et al., 1994). More recently, the engineering of the RGD sequence into an exposed loop of the adenovirus fiber protein resulted in enhanced infection and a broadened host range (Dmitriev et al., 1998). Finally, cryo-EM studies have been employed to examine the interaction of $\alpha_v\beta_5$ with the penton base (Chiu et al., 1999). The specific roles of each integrin in the attachment, internalization, and penetration of the endocytic vesicle by the virus may vary in different cell types (Chiu et al., 1999).

Small molecule antagonists of RGD–integrin interactions have been synthesized through a medicinal chemistry approach, and have been previously shown to inhibit vitronectin binding to $\alpha_v\beta_3$, cell adhesion to fibrinogen, bFGF-induced endothelial cell proliferation, corneal neovascularization, tumor growth in vivo and bone resorption in vitro and in vivo (Engleman et al., 1997; Carron et al., 1998). This paper identifies these low-molecular weight synthetic molecules as antagonists of adenovirus infection in cell culture.

2. Materials and methods

2.1. Peptides and peptidomimetics

SC-68448 has been described previously (Carron et al., 1998). All peptidomimetics were synthesized in the Medicinal Chemistry Group at Pharmacia/Searle (Fig. 1). Peptides GRGDSP and GRGESP were obtained from Bachem (King of Prussia, PA).

2.2. Cells

Human embryonic kidney 293 cells were obtained from the laboratory of Jeff Smith (Burnham Institute, San Diego, CA). HeLa cells were

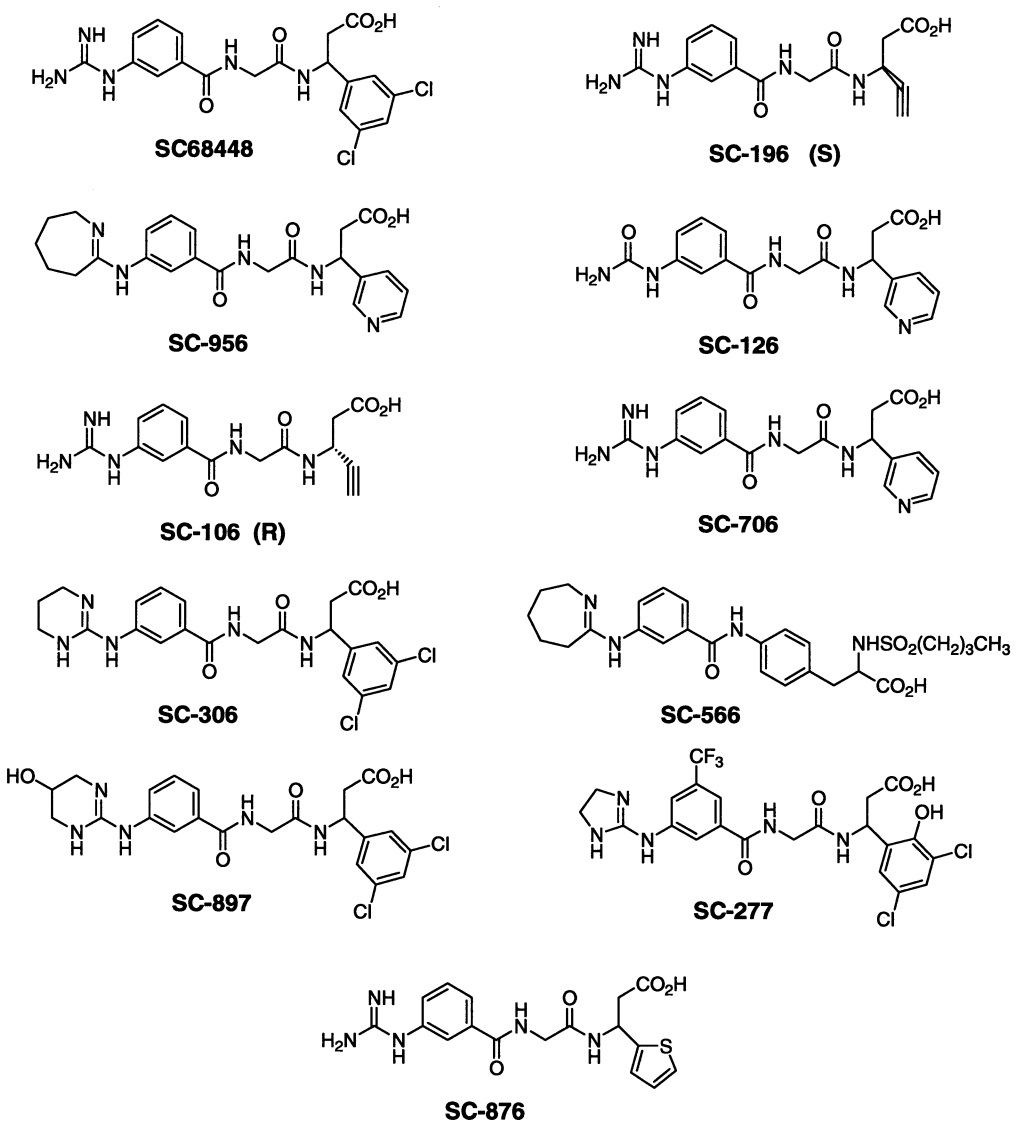

Fig. 1. Structures of RGD peptidomimetic compounds used in this study.

obtained from ATCC (Manassas, VA). A derivative of 293 cells that expresses high levels the $\alpha_v\beta_5$ integrin (293/β5) has been previously described (Engleman et al., 1997). Cells were cultured in Optimem (Life Technologies, Gaithersburg, MD) containing 10% calf serum.

2.3. Virus

Human adenovirus type 2 was obtained from ATCC (Manassas, VA). Virus was prepared on 293/β5 cells as described (Graham and Prevec, 1991). The $TCID_{50}$ on 293/β5 cells was determined by the method of Reed and Muench (Reed and Muench, 1938). Stocks contained 10^9–10^{10} $TCID_{50}$ per ml. Adenovirus type 2 containing the RGD/RGE substitution in the penton base sequence (Bai et al., 1993) was provided by Dr Paul Freimuth (Brookhaven National Laboratory, Upton, NY).

2.4. Infectivity assays

Infection assays conducted in the presence of RGD peptides or compounds were conducted essentially as described by Wickham et al. (Wickham et al., 1993). Cells (2×10^6) were dissociated non-enzymatically using Cell Dissociation Solution (Sigma, St. Louis, MO) and resuspended in 2.0 ml Optimem (Life Technologies, Gaithersburg, MD). RGD peptides, compounds or vehicle (dimethyl sulfoxide or water) were added to obtain the concentrations noted in the figure legends, and incubations were performed for 60 min at 4 °C to prevent internalization of the integrin prior to adenovirus addition. Adenovirus was added (in suspension) at a MOI of approximately 1 for an additional 60 min at 4 °C, and the cultures were shifted to 37 °C for 30 min. Cells were treated with trypsin (0.25 mg/ml in PBS without cations) to inactivate the virus that was not internalized by the cell. This resulted in release of DNA that contributed to the viscosity of the cell suspension. DNAse (RQ, Promega, Madison, WI) treatment at 37 °C for 10 min decreased the viscosity. Cells were concentrated by centrifugation, resuspended in 2.0 ml of Optimem/10% fetal calf serum, added to poly-lysine treated wells of a 24-well dish (2×10^6 cells per well), and cultured for approximately 44 h at 37 °C. For visualization of infected cells, the cells were fixed in 90% methanol/10% PBS and were washed twice in PBS/gelatin (Sigma, St. Louis, MO). Infected cells were detected using monoclonal antibody MAB 8051 (Chemicon International, Temecula, CA) and FITC-conjugated goat anti-mouse (62-6311, Zymed Laboratories, South San Francisco, CA). The anti-adenovirus antibody was raised against Ad3 and cross-reacts against 41 adenovirus serotypes. Fluorescent cells were visualized and counted by two independent observers using an Olympus fluorescent microscope. Statistical analysis (Student's and Mann–Whitney tests) was performed using the INSTAT Software package (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Peptidomimetics of RGD inhibit adenovirus infection in a dose-dependent manner

Human embryonic kidney 293 cells constitutively express the $\alpha_v\beta_1$ integrin, but little or no other alpha v-containing integrins (Bodary and McLean, 1990). These cells were transfected with plasmid DNA to over express the β_5 subunit, thus making $\alpha_v\beta_5$ the predominant integrin on the cell surface (293/β5). Previous studies have shown that overexpression of β1 or β5 enhances the susceptibility of 293 cells to infection by adenovirus (Wickham et al., 1994; Huang et al., 1995; Hashimoto et al., 1997). Human 293 cells and 293/β5 cells were incubated in the presence of 10 or 100 uM concentrations of the RGD peptidomimetic compound SC-68448 (Fig. 1) prior to adenovirus infection. Approximately 40 h post-infection, the cells were fixed and viral antigen was detected using an anti-adenovirus monoclonal antibody and a FITC-tagged goat anti-mouse antibody. Little or no fluorescence was observed at earlier time points suggesting that the antibody was detecting newly synthesized antigen, not input virus (data not shown). The results indicated that treatment of cells with SC-68448 decreased aden-

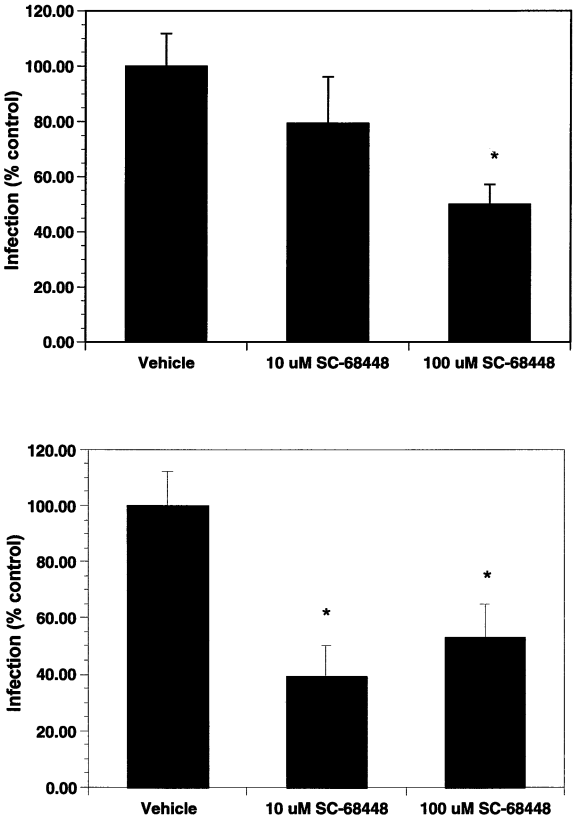

Fig. 2. Integrin antagonists reduce adenovirus infection in human cells. Human 293 (A) or 293/β5 (B) cells were preincubated with SC-68448 at concentrations of 10 or 100 uM prior to infection with adenovirus as described in Section 2. Infected cells were detected by immunostaining using an anti-adenovirus monoclonal primary antibody and a goat-anti-mouse-FITC tagged secondary antibody. Fluorescent cells were counted using a microscope and the mean value from four wells was expressed as the percent of fluorescent cells in wells treated with the vehicle control. Values represent the mean ± S.D. These are results from a single experiment from at least two independent determinations with similar results. *, $P < 0.01$ vs. vehicle using two-tailed Student t-test.

ovirus antigen production on 293 cells in a dose-dependent manner (Fig. 2A). In 293/β5 cells, SC-68448 treatment produced maximal inhibition of about 50% at both 10 and 100 uM (Fig. 2B), suggesting that these cells over expressing $\alpha_v\beta_5$ may be more sensitive to compound inhibition of adenovirus infection. To further examine the dose-dependency of the effect and to determine the maximal level of inhibition, 293/β5 cells were treated with 20, 200 or 2000 uM SC-68448 or 2000 uM of the peptide G**RGD**SP or G**RGE**SP. These peptides represent positive and negative controls, respectively, with previously characterized effects on adenovirus infection in cultured cells (Wickham et al., 1993). There was a statistically significant reduction in the number of fluorescent cells in all SC-68448 treated wells and in the G**RGD**SP treated wells (Fig. 3). Maximum inhibition of about 50% was achieved with 200 uM SC-68448. Likewise, 2000 uM G**RGD**SP resulted in 50% inhibition. This level of inhibition is consistent with previous data using RGD peptides to inhibit adenovirus internalization (Wickham et al., 1993). These data suggest that the RGD–integrin interaction contributes to, but is not essential for, virus replication, and that other interactions are also likely to be important for infectivity.

The organic compound SC-68448 and RGD peptide inhibited adenovirus infection similarly, thus implying these effects are due to disruption

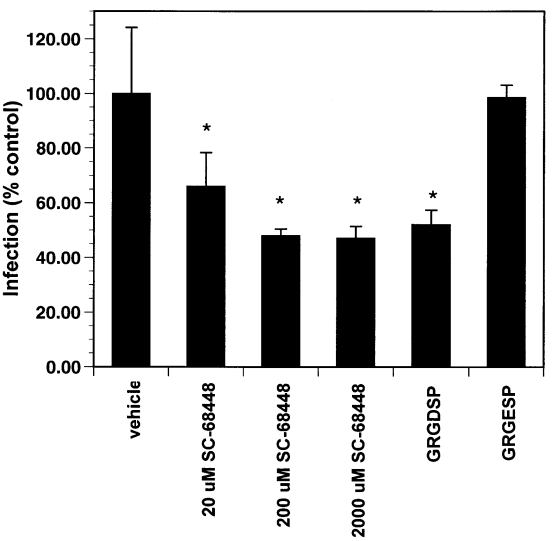

Fig. 3. Comparative potency of an RGD-containing peptide and RGD peptidomimetic. 293/β5 cells were preincubated with various concentrations of SC-68448 or a single concentration of synthetic peptides (2000 uM). Cells were then infected with adenovirus and assayed as described in Fig. 2. Fluorescent cells were counted using a microscope and the mean value from four wells was expressed as the percent of fluorescent cells in wells treated with the vehicle control. Values represent the mean ± S.D. *, $P < 0.05$ vs. vehicle using two-tailed Mann–Whitney test.

of the RGD–integrin interaction and not to compound-associated cytotoxicity. Indeed in control experiments performed in the absence of virus, we have found no significant loss of viability ($< 5\%$) of 293/β5 or other cell types caused by incubation with compounds at concentrations equal to or exceeding those described in this report (data not shown). Furthermore, previous studies have shown that exposure of cells for several days with up to 1 mM SC-68448 has no significant effect on proliferation (Carron et al., 1998, data not shown). Nevertheless to further confirm the mechanism of the inhibitory activity, several integrin antagonists of different chemical structures and potencies in integrin functional assays, unrelated to adenovirus infection, were examined (Fig. 1). Each of these known RGD peptidomimetics inhibited adenovirus infection to a similar degree at 100 uM concentration (Fig. 4a). This further supports the concept that the inhibition of infection is via disruption of the RGD–integrin interaction and not via cell toxicity or other nonspecific property of any one particular compound such as SC-68448. Finally, we tested an additional set of peptidomimetic agents to examine whether the interaction of cellular integrins with the virus is qualitatively similar to the interaction with another RGD-containing ligand, the extracellular matrix protein vitronectin (Fig. 4B, Fig. 1). The rank order potency of inhibition of adenovirus infection was approximately the same as that observed in independent assays of $\alpha_v\beta_3$-dependent cell adhesion to vitronectin.

3.2. Adenovirus with a mutated penton base is not inhibited by SC-68448

Adenovirus infection does not require penton base/integrin interaction for infection, however, this interaction does appear to enhance the infectivity. Bai et al. (Bai et al., 1993) have mutated the penton base protein, substituting RGE for the RGD motif, and found that this mutation delayed virus replication. Since Ad-2 RAE cannot utilize the RGD–integrin interaction, we expected that there should be no effect of known integrin antagonists on the ability of this virus to infect cells. The mutated Ad-2 RAE was used to infect cells

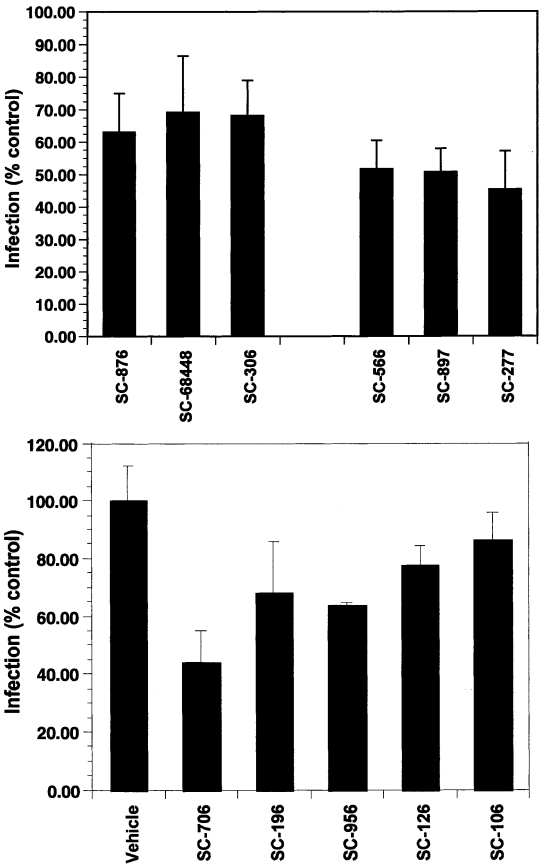

Fig. 4. Anti-viral effects of integrin antagonists derived from different chemical classes and with varying potency for vitronectin adhesion. (A) 293 cells were treated with 100 uM antagonists having a variety of chemical structures and assayed as in Fig. 2. The fluorescent cells were counted using a microscope and the mean value from four wells was expressed as the percent of fluorescent cells in wells treated with the vehicle control (water, except for SC-566, SC-897 and SC-277 which were solublized in DMSO). Values represent the mean $\pm$ S.D. All results were statistically significant relative to vehicle controls ($P < 0.03$ using two-tailed Mann–Whitney test). (B) HeLa cells were preincubated with the indicated compounds at 100 uM, and the effect on viral infection was determined as in Fig. 2. Compounds are shown ordered from left to right according to decreasing potency (IC_{50} range = 1.1–38.8 nM) in an independent assay of $\alpha_v\beta_3$ function based on 293 cell binding to vitronectin.

treated with SC-68448, and in contrast to the results obtained with wild type Ad-2, no inhibition of infection was observed (Fig. 5). These data strongly suggest that the mechanism of anti-viral action exhibited by SC-68448 and other chemical

compounds is specific and dependent upon RGD-mediated interactions between the penton base and integrins.

4. Discussion

Adenovirus infections cause a variety of illnesses including gastrointestinal, respiratory and ocular diseases. These infections are typically limited but can have severe consequences in immunocompromised patients such as those undergoing transplantation or HIV infected individuals (Ribaud et al., 1999). While some progress has been made, more effective treatments are needed (Gordon, 2000; Legrand et al., 2001). Furthermore, reports of a gene therapy fatality when using adenoviral vectors (Mickelson et al., 2002) suggest that, perhaps, effective fast acting therapeutics that block internalization could be a benefit. Currently used compounds that are effective against adenoviruses are typically nucleotide or nucleoside analogues that interfere with DNA replication (Mentel et al., 1997; Ribaud et al., 1999), or Shiff base analogs of aminohydroxyguanidine that could be interfering with ribonucleotide reductase (Das et al., 1999). Clearly there is a need for further development of antiviral compounds directed against adenoviruses, and another potential approach for the development of such agents is described in this report. This approach involves inhibiting the integrin-mediated internalization of adenovirus, an early step in the replication cycle.

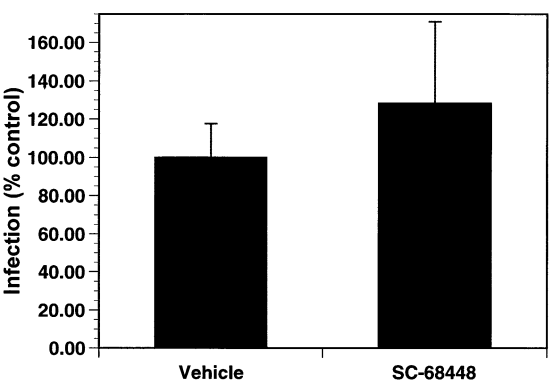

Fig. 5. Adenovirus lacking the RGD sequence is insensitive to SC-68448. 293 cells were preincubated with SC-68448 (100 uM) prior to infection by Ad-2 RAE. Infection was examined as in Fig. 2. Fluorescent cells were counted using a microscope and the mean value from four wells was expressed as the percent of fluorescent cells in wells treated with the vehicle control. Values represent the mean $\pm$ S.D. These are results from a single experiment from at least two independent determinations with similar results. The difference between vehicle and compound-treated cells was not statistically significant ($P = 0.35$ by two-tailed Student t-test).

Binding of adenovirus to a cell surface receptor is the first step in the viral replication cycle. The most characterized receptor is the coxsackie and adenovirus receptor (CAR) protein on the cell surface. More recently, the interaction of the fiber protein and CAR has been examined by crystallography (Bewley et al., 1999). Results suggest that a high affinity interaction occurs between three CAR domains per fiber trimer. Moreover, mutagenesis experiments have shown that mutations in the fiber that interface with CAR can change the tropism of the virus (Roelvink et al., 1999). It has been previously shown that adenovirus infection is enhanced by interactions between the penton base of the adenovirus and integrins of the α_v family expressed on the cell surface. Specifically, this interaction appears to enhance the internalization of the virus. The adenovirus penton base protein has an RGD motif that is commonly found in proteins that interact with integrins. While the interaction of proteins with the RGD motif and various integrins have been fairly well characterized, small molecule peptidomimetic antagonists with selective potency against $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have only more recently been described (Engleman et al., 1997; Carron et al., 1998; Holzemann, 2001). Inhibitory compounds, some with oral bioavailability, have been designed which are potent inhibitors in a variety of in vitro and in vivo assays of α_v integrin functions, but which do not substantially interfere with platelet aggregation and blood clotting which is mediated by the closely related integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIbIIIa). Given that a large and growing number of microorganisms appear to use host cell integrins as a primary binding target or as an accessory protein, experiments were performed to determine whether these small molecule antagonists were active in models of pathogen infection.

Integrin antagonist SC-68448 was demonstrated to inhibit infection of cultured human embryonic kidney cells with the adenovirus-2 serotype in a dose-dependent manner. The degree of inhibition (approximately 50%) was similar to that achieved with the peptide G**RGD**SP. The conclusion that this effect is mediated by specific competition with the RGD sequence in the viral penton base protein, and not due to compound-associated toxicity, is supported by the following results: First, SC-68448 did not inhibit the infection of a mutated adenovirus lacking the RGD sequence; second, peptidomimetic compounds from several chemical classes were capable of mediating the effect; finally, the rank order potency of a group of compounds for inhibiting viral infection recapitulated the rank order potency observed in independent assays of $\alpha_v\beta_3$ function (e.g. $\alpha_v\beta_3$-dependent cell binding to the classic RGD-containing ligand vitronectin). The latter result also suggests that the structural nature of the penton base RGD interaction with the integrin is not dramatically different than that of RGD contained in extracellular matrix proteins such as vitronectin. This is interesting considering the lack of substantial homology in the amino acid sequences flanking the RGD, and the very different functional roles of the viral protein and extracellular matrix constituent.

Our studies demonstrate that RGD peptidomimetic molecules that are being widely investigated as potential therapies for cancer, osteoporosis and cardiovascular disorders (Hartman and Duggan, 2000; Holzemann, 2001; Kerr et al., 2000), may also have utility in modifying infectious disease. Although our alpha v integrin antagonists produced somewhat modest efficacy (50% inhibition) in our tested experimental system with adenovirus, the effects were specific, and may prove to be synergistic with other treatment regimens employed clinically. We speculate that RGD peptidomimetics may have greater impact in other pathogen–host cell interactions. For example, RGD mutations in the Foot and Mouth Disease Virus can result in almost complete loss of infectivity in BHK cells (Mason et al., 1994). Compounds with broader integrin selectivity than those employed here (e.g. against $\alpha5\beta1$ and IIbIIIa) may prove to be beneficial depending on the particular pathogen and the particular integrins with which it is capable of interacting on the target host cells. Given the widespread exploitation of cell surface integrins by invading microorganisms, synthetic integrin antagonists may be useful chemotherapeutic agents for controlling infection especially in situations where vaccines do not exist or pathogens resistant to current therapies arise. In addition, adenoviral vectors have been used to deliver genes of interest to tissues in vivo. Synthetic molecules that selectively regulate RGD:integrin interactions may be a means of decreasing gene delivery to inappropriate tissues.

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