ELSEVIER

Effects of RGD Peptides on Cells Derived From the Human Eye

Yoshiko Imoto*, Nobuyuki Ohguro*, Atsushi Yoshida*,
Motokazu Tsujikawa*, Yoshitsugu Inoue† and Yasuo Tano*

*Department of Ophthalmology, Osaka University Medical School, Osaka, Japan;
†Department of Ophthalmology, Tottori University Faculty of Medicine, Tottori, Japan

Purpose: To investigate the effects of Arg-Gly-Asp (RGD) peptides on cells derived from the human eye.

Methods: SV40-transformed human corneal epithelial cells (SCE), SV40-transformed human lens epithelial cells (SLE), normal human keratocytes (NK), and human retinal pigment epithelial cells stably expressing human telomerase reverse transcriptase (TRPE) were used. A human corneal cDNA library was screened to isolate the human homologue of p130 Crk-associated substrate (Cas). Next, after these cells were attached to the substratum, RGD-containing soluble tetrapeptides Arg-Gly-Asp-Ser (RGDS) were added to the culture medium and morphological changes were observed.

Results: A clone, H-2, consisting of 3228 nucleotides, with a long open reading frame (870 amino acid residues) was isolated, and determined to be the human homologue of Cas. After addition of the RGDS peptides, both SLE and TRPE detached from the plastic culture plate, but SCE and NK did not. Detached SLE and TRPE showed decreased levels of tyrosine phosphorylation in Cas.

Conclusions: These results indicated that the signaling pathway through Cas played an important role in epithelial cell adhesion in the eye. **Jpn J Ophthalmol 2003;47:444–453**

Key Words: Cell adhesion, focal adhesion kinase, intracellular adhesion molecules, p130 Crk-associated substrate, tetrapeptides Arg-Gly-Asp-Ser.

Introduction

Adhesion of cells to the extracellular matrix (ECM) is primarily mediated by the integrin receptors.[1] Cell–ECM interactions transmit extracellular signals into the cell interior via the integrins,[2] and intracellular signaling has been shown to involve tyrosine phosphorylation of intracellular proteins. Among these proteins, tyrosine phosphorylation of the nonreceptor tyrosine kinase, focal adhesion kinase (FAK),[3,4] is increased by diverse signaling molecules that regulate cell migration, proliferation, and cell survival.[5] FAK associates with a number of different signaling proteins enabling protein-tyrosine kinases to couple to a variety of different downstream signaling pathways.[6,7] Previous studies have suggested that p130 Crk-associated substrate (Cas)[8] plays a key role in the signaling pathway through FAK.[9] The association of Src-family proteins with FAK at sites of integrin clustering can also potentiate the association and tyrosine phosphorylation of Cas, which is important for integrin-mediated signal transduction leading to cell shape change and migration.[10]

Synthetic peptides containing the Arg-Gly-Asp (RGD) sequence have been recognized as inhibitors of fibronectin-mediated cell attachment, because the RGD motif is an integrin recognition motif found in fibronectin.[11–13] In the ophthalmology field, it has also been reported that RGD-containing peptides can inhibit the adhesion of some epithelial cell types to its substrates. Several researchers have attempted the clinical application of RGD-containing peptides for the prevention of secondary cataract formation and proliferative vitreoretinopathy.[14–18]

In the present study, first we identified the human homologue of Cas, because it had not yet been identified.

Received: September 30, 2002
Correspondence and reprint requests to: Nobuyuki OHGURO, MD, Department of Ophthalmology, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Published by Elsevier Science Inc.

0021-5155/03/$–see front matter
doi:10.1016/S0021-5155(03)00135-7

As the next step, we confirmed that RGD peptides inhibit the adhesion of lens epithelial cells and retinal pigment epithelial cells, but not corneal epithelial cells and keratocytes. For a better understanding of the events underlying the different effects of cell–matrix interactions by RGD peptides in each cell type, we examined the levels of tyrosine phosphorylation of FAK and Cas in each cell type to investigate the effects of RGD peptides on the intracellular signaling pathways. In addition, we further analyzed the expression patterns of the integrin subunits in each cell type to investigate the possible mechanisms for these alterations by the RGD peptides.

Materials and Methods

Cell Lines

SV-40 transformed human corneal epithelial cells (SCE) were a kind gift from Dr. Sasaki-Araki.[19] Normal human keratocytes (NK) were a kind gift from Dr. Sai.[20] To immortalize human lens epithelial cells, cells were isolated from human lenses of infants who had undergone treatment for retinopathy of prematurity. Isolated cells were infected with a recombinant SV40-adenovirus vector as previously reported,[21] and established as SV40-transformed human lens epithelial cells (SLE). Informed consent was obtained from the parents of these patients in accordance with the Declaration of Helsinki. Human retinal pigment epithelial cells stably expressing human telomerase reverse transcriptase (TRPE) were purchased from Clontech Laboratories (Palo Alto, CA, USA).

Antibodies

Polyclonal anti-human FAK antibody (FAK-Ab), polyclonal anti-rat C-terminal Cas antibody(Cas C-20), polyclonal anti-rat N-terminal Cas antibody (Cas N-17), monoclonal anti-phosphotyrosine antibody (PY20), and secondary antibodies labeled with horseradish peroxidase (anti-rabbit IgG, anti-mouse IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies against human α2, α3, α5, and αv integrins, and the anti-mouse IgG1-FITC antibody were purchased from PharMingen (San Diego, CA, USA).

Chemicals

Dulbecco's Modified Eagle Medium (DMEM), Ham F-12, fetal bovine serum (FBS) and 0.05% trypsin-0.025% EDTA were purchased from Nikken Bio Medical Labware (Kyoto). The soluble synthetic tetrapeptides Arg-Gly-Asp-Ser (RGDS)[22] and the control peptides Arg-Gly-Glu-Ser (RGES) were purchased from Invitrogen (Carlsbad, CA, USA). Ten-centimeter culture plates, 75-cm^2 culture flasks and 6-well culture plates were obtained from Corning (Corning, NY, USA). The human corneal cDNA library was purchased from Stratagene (La Jolla, CA, USA). The protease inhibitors leupeptin and aprotinin were obtained from Roche Diagnostics (Mannheim, Germany). Sodium orthovanadate was purchased from Sigma Chemical. (St. Louis, MO, USA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals were purchased from Nacalai tesque (Kyoto).

Cell Culture

SCE were grown in 75-cm^2 culture flasks in supplemented hormone epithelial medium (SHEM: 15% FBS, 5 μg/mL insulin, 0.1 μg/mL cholera toxin, 10 ng/mL epidermal growth factor [EGF], 40 μg/mL gentamicin in DMEM/Ham F-12) and maintained in a humidified 37ºC incubator containing 5% CO_2. NK were cultured in DMEM/Ham F-12 with 15% FBS. SLE were cultured in DMEM with 20% FBS. TRPE were cultured in DMEM/Ham F-12 with 10% FBS, 0.3% sodium bicarbonate, and 2 mM L-glutamine. The medium was changed every third day until growth was confluent. The cells were then harvested by trypsinization and replated (passage dilution 1:4).

Western Blot Analysis

SCE, NK, SLE, and TRPE were lysed with lysis buffer [50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol, 400 μM sodium orthovanadate, 1% NP-40, 100 μg/mL phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 8 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 μg/mL pepstatin] at 4ºC for 20 minutes. The cell lysates were centrifuged to remove insoluble material at 12,000 *g* for 10 minutes. The supernatants were replaced into sterilized microtubes and protein concentrations were evaluated using the DC protein assay kit (Bio-RAD, Richmond, CA, USA). Each protein sample (10 μg) was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to a polyvinylidene difluoride (PVDF) membrane, and immunoblotted with Cas C-20 or Cas N-17 primary antibodies. Horseradish peroxidase-conjugated IgG was used as the secondary antibody and the ECL kit was used for detection.

Isolation of Human Cas cDNA

A human corneal cDNA library was generated with the Uni-Zap XR vector (Stratagene) and immunoscreened using Cas C-20. The detailed protocol for isolating the cDNAs is described in the instruction manual for the ZAP-cDNA Synthesis Kit protocol. Four positive clones were

Jpn J Ophthalmol
Vol 47: 444–453, 2003

isolated and the DNA sequences determined with the ABI PRISM™ 310 Genetic Analyser (Applied Biosystems, Forester City, CA, USA).

Northern Hybridization Analysis

Poly(A)$^+$RNA was extracted from both SCE and NK, and 2 ug of each poly(A)$^+$RNA was subjected to electrophoresis on a 1% formaldehyde-agarose gel and transferred onto a Hybond N$^+$ membrane. Commercially available Northern blots containing mRNA of multiple human tissues were used (Human Normal Tissue mRNA blot 1, Normalized Type; Toyobo, Osaka). Hybridization was performed using an approximately 1.0-kbp fragment of the isolated clone created by reverse transcriptase-polymerase chain reaction as a probe (primer:sense 5′-CTGGGAACCGCCTCAAGATC-3′,308-320; anti-sense 5′-GGTCGACAGTGGTGTGTATG-3′,1353-1372; GenBank Accession No. AB040024).

Microscopic Observation of the Effect of RGDS Peptides on the Adhesion of Ocular Cells

Each ocular cell type was seeded in 6-well plastic culture plates (2×105 cells/well). After 24 hours, 1 mM RGDS or RGES peptides was added to the culture medium and the status of cell attachment was observed at 0.5 hours, 2 hours, and 24 hours thereafter.

Protein Extraction

Each cell type was seeded on 10-cm plastic culture plates (1×106 cells/plate) and incubated for 24 hours. RGDS or RGES peptides (1 mM) was then added to the culture medium. After 30 minutes, cell lysates were prepared as for Western blot analysis.

Immunoprecipitation and Immunoblotting

Cell lysates (containing 400 μg of protein) were precleared with normal rabbit IgG and protein-A-agarose for 30 minutes at 4ºC and centrifuged at 12,000 *g* for 1 minute. Supernatants were reacted with FAK-Ab or Cas C-20 at 4ºC for 1 hour and incubated with protein-A-agarose overnight. The immunoprecipitates were washed four times with lysis buffer, and 30 μL of $2 \times$ SDS-PAGE sample buffer was added. After 2 minutes at 100ºC, equal volumes of immunoprecipitates were run on a 7.5% SDS-PAGE gel, electrotransferred to a PVDF membrane, and immunoblotted with PY20. FAK-Ab and Cas C-20 were also used to quantify the amount of FAK and Cas protein, respectively. The membrane was incubated with horseradish peroxidase-linked anti-rabbit IgG and developed using the ECL kit according to the manufacturer's instructions.

The phosphorylated protein was quantified with NIH Image software. Analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Quantification of the Expression of Integrins

The expression of α integrin on the surface of each cell type was analyzed by flow cytometry. Briefly, culture cells were detached from culture plates using 2 mM EDTA, and each cell type (5×105 cells) was incubated with monoclonal antibodies against human α2, α3, α5, αv integrin, or isotype matched controls followed by anti-mouse IgG-FITC. Flow cytometric analysis of 1×104 cells from each sample was performed on the FACS Calibur TM flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) according to standard procedures.

Results

Each experiment was performed at least three times, and the results were consistent. The data represent typical results obtained.

Human Cas Protein Expression

We first detected that the anti-rat C-terminal Cas antibody cross-reacted with a protein from SCE, NK, SLE, and TRPE cells from human ocular tissues by Western blotting, since a single band appeared in each lane on the membrane (Figure 1A). The molecular weight of this protein was approximately 120 kDa. We suspected that this protein must be the human homologue of Cas and attempted to identify this protein as mentioned in the next section. On the other hand, multiple bands appeared using Cas N-17 (data not shown). From these results we concluded that Cas C-20 should be used for the analysis of the human homologue of Cas.

Human Homologue of Cas

Four positive clones were isolated upon immunoscreening of a human corneal cDNA library using Cas C-20. Among these clones, clone H-2 consisted of 3228 nucleotides (Figure 2A; GenBank Accession No. AB040024), and had a long open reading frame (870 amino acid residues). At position 142, an initiation codon is flanked by sequences matching Kozak's criteria.[23] The open reading frame is flanked at the 3′ end by a translation termination codon (TGA) at position 2752. A consensus polyadenylation signal, AATAAA, is located at sites 3211-3216 in the 3′-untranslated region upstream of the

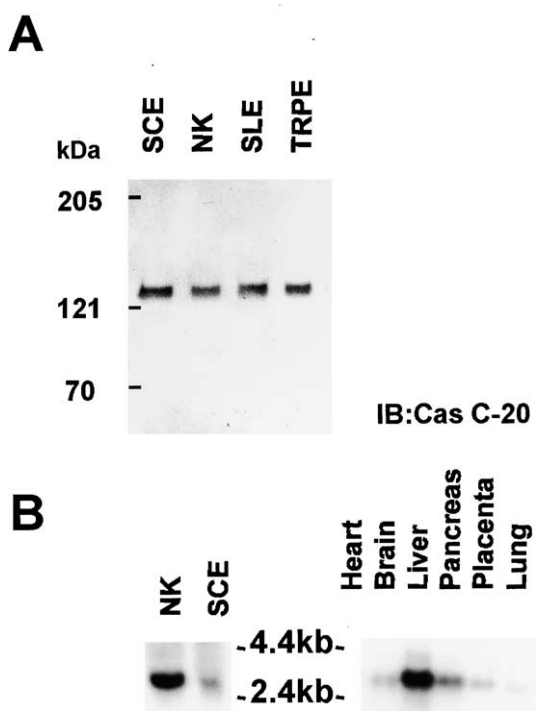


Figure 1. (**A**) The expression of human p130 Crk-associated substrate (Cas) protein in cells derived from ocular tissue. Protein (10 μg) from each cell type was analyzed by Western blotting with the anti-rat C-terminal Cas antibody. Size markers are indicated. (**B**) Northern blot analysis of the expression of H-2 in normal human keratocytes (NK) and SV40-transformed human corneal epithelial cells (SCE) from ocular tissue (left panel), and in various human tissues (right panel). Size markers are indicated. SLE: SV40-transformed human lens epithelial cells, TRPE: telomerase reverse transcriptase.

poly(A) tail. A search for homologous proteins revealed that the amino acid sequence of the H-2 protein had extensive homology (90%) with the rat and mouse Cas protein (Figure 2B). Taken together, we concluded that the H-2 protein is the human homologue of adaptor protein Cas. By Northern blot analysis, both SCE and NK displayed a single transcript of approximately 3.3 kb (Figure 1B, left panel). A mRNA product of the same site was also found to be present in all human tissues tested, being most abundant in the liver (Figure 1B, right panel).

Recently, Brinkman et al reported on the identification of the breast cancer anti-estrogen resistance 1 protein (BCAR1),[24] and the amino acid sequence of BCAR1 was found to be identical to that of the H-2 protein.

Effects of RGDS Peptides on Adhesion of Ocular Cells

To examine the effects of RGDS peptides on the adhesion of ocular cells, RGDS peptides (1 mM) were added to the culture medium without serum. Within 30 minutes after addition of the RGDS peptides, both SLE and TRPE began to detach from the plastic plate and by 2 hours, they had lost contact with the plate completely (Figure 3). After 24 hours however, these cells had become reattached to the plates. On the other hand, neither SCE nor NK detached significantly from the plates throughout the observation period. The presence of RGES peptides did not cause any of the cell types to detach from the substratum (Figure 4).

Effects of RGDS Peptides on Tyrosine Phosphorylation

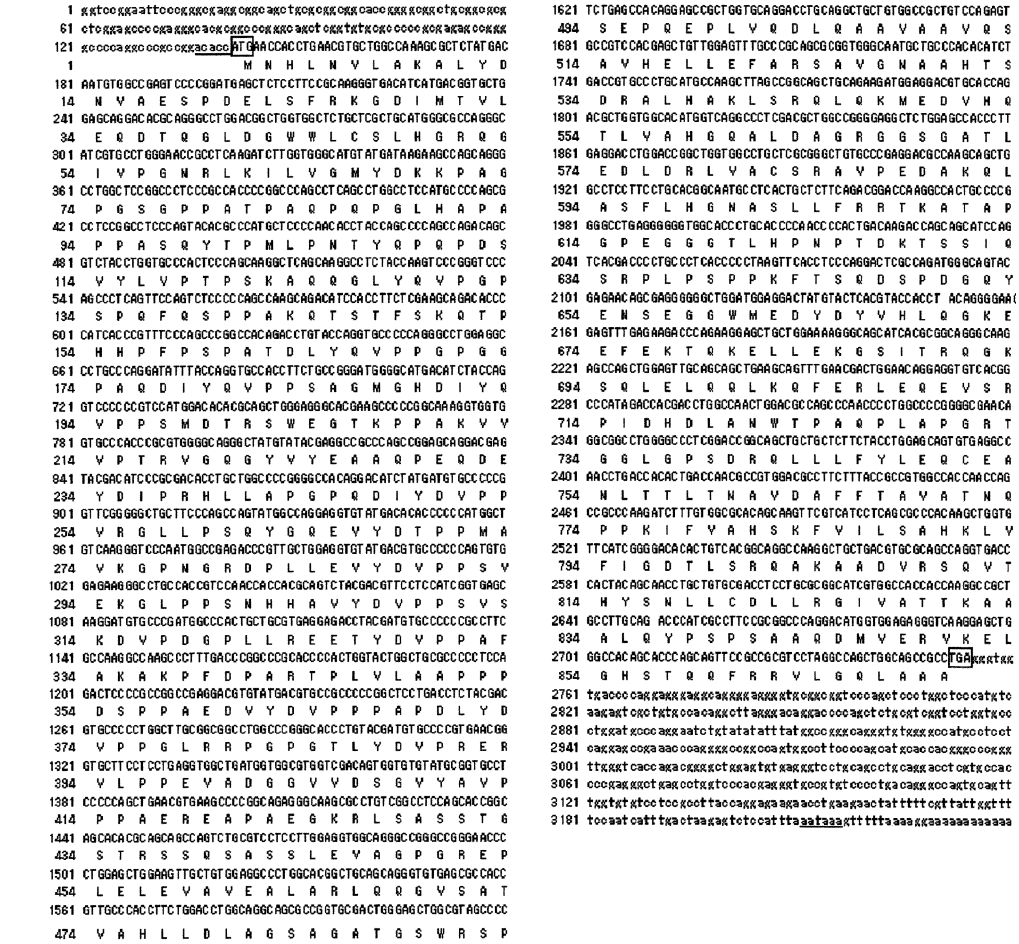
We next tested whether there was a change in the level of tyrosine phosphorylation of FAK and Cas after the addition of RGDS peptides because tyrosine phosphorylation of FAK has been shown to be an essential event in focal adhesion formation.[5] The amount of protein immunoprecipitated with the FAK-Ab or Cas C-20 and stained by PY20, phosphorylated FAK or Cas, was dramatically decreased in SLE and TRPE by the addition of RGDS peptides but not in SCE and NK (Figure 5). The amounts of FAK and Cas themselves, induced by bands stained with anti-FAK and Cas C-20, were not affected in any cells by the addition of RGDS peptides. Thus, these results clearly demonstrate that RGDS peptides induce a marked decrease in the level of tyrosine phosphorylation of FAK and Cas in SLE and TRPE. RGES peptides, on the other hand, did not affect the degree of tyrosine phosphorylation of the ocular cells (data not shown). Figure 6 summarizes the effects of RGDS/RGES peptides on the level of tyrosine phosphorylation of FAK and Cas in each cell type.

Expression Pattern of Integrin Subunits

We hypothesized that the different responses among cells derived from the human eye to RGDS peptides may be due to different expression patterns of the integrin subunits. To test this hypothesis, we used flow cytometry to investigate the expression levels of the α2 subunit, which is thought to be a major membrane receptor for collagen, the α3 subunit, which is thought to be a major membrane receptor for vitronectin, and the α5 and αv subunits, which are believed to be major receptors for fibronectin. The results showed that while both SCE and NK expressed the α2 subunits dominantly, both

Figure 2. (**A**) The nucleotide sequence of human H-2 (GenBank No. AB040024). (**B**) Comparison of human H-2 with rat and murine p130 Crk-associated substrate (Cas). A comparison of the amino acid sequence of human H-2 with rat and murine Cas is shown.

A



B

```
human-H-2  MNHLNVLAKALYDNVAESPDELSFRKGDIMTVLEQDTQGLDGWWLCSLHGRQGIVPGNRL  60
rat-Cas    -KY-----------------------------------R-------------------------  60
murine-Cas -KY-----------------------------------R-------------------------  60

human-H-2  KILVGMYDKKPAGPGSGPPATPAQPQP    GLHAPAPPASQYTPMLPNTYQPQPDSVYL 116
rat-Cas    -----------A-P-------P----SLPQ-V-T-V------S----TA----P-N--- 120
murine-Cas -----------G-P-------P----SLPQ-V-A-V------S----TA----S-N--- 120

human-H-2  VPTPSKAQQGLYQVPGPSPQFQSPPAKQTSTFSKQTPHHPFPSPATDLYQVPPGPGGPAQ 176
rat-Cas    ------T-------A---N--------------------S---------------S--- 180
murine-Cas ------T-------A---N--------------------S---------------S--- 180

human-H-2  DIYQVPPSAGMGHDIYQVPPSMDTRSWEGTKPPAKVVVPTRVGQGYVYEAAQPEQDEYDI 236
rat-Cas    ----------T----------L---S-------------------S-A------T 240
murine-Cas ----------I----------L---G-------------------A-T------T 240

human-H-2  PRHLLAPGPQDIYDVPPVRGLLPSQYGQEVYDTPPMAVKGPNGRDPLLEVYDVPPSVEKG 296
rat-Cas    --------S--------------N----------------------D---------- 300
murine-Cas --------P--------------N----------------------D---------- 300

human-H-2  LPPSNHHAVYDVPPSVSKDVPDGPLLREETYDVPPAFAKAKPFDPARTPLVLAAPPPDSP 356
rat-Cas    -PP-N--S-------------------------------P-----T-H--I--------- 360
murine-Cas -LS-S--S-------------------------------P-----T-H--I--------- 360

human-H-2  PAEDVYDVPPPAPDLYDVPPGLRRPGPGTLYDVPRERVLPPEVADGGVVDSGVYAVPPPA 416
rat-Cas    P-------------------------------------------S-I-D--------- 420
murine-Cas A-------------------------------------------S-V-D--------- 420

human-H-2  EREAPAEGKRLSASSTGSTRSSQSASSLEVAGPGREPLELEVAVEALARLQQGVSATVAH 476
rat-Cas    -----TD-------------------VV----------T---------T---- 480
murine-Cas -----TD-------------------VV----------S---------T---- 480

human-H-2  LLDLAGSAGATGSWRSPSEPQEPLVQDLQAAVAAVQSAVHELLEFARSAVGNAAHTSDRA 536
rat-Cas    ----V---SGP-G--ST------PV---K------HG----------S--SS-T-----T 540
murine-Cas ----V---SGP-G--GT------PA---K------HG----------G--SN-T-----T 540

human-H-2  LHAKLSRQLQKMEDVHQTLVAHGQALDAGRGGSGATLEDLDRLVACSRAVPEDAKQLASF 596
rat-Cas    ---------------Y----V---V--S---GP-F-LD---------------------- 600
murine-Cas ---------------Y----V---V--S---SP-F-PE---------------------- 600

human-H-2  LHGNASLLFRRTKATAPGPEGGGTLHPNPTDKTSSIQSRPLPSPPKFTSQDSPDGQYENS 656
rat-Cas    --------------PG-----SSS--L-----A---------------------------- 660
murine-Cas --------------PG-----SSS--P-----A---------------------------- 660

human-H-2  EGGWMEDYDYVHLQGKEEFEKTQKELLEKGSITRQGKSQLELQQLKQFERLEQEVSRPID 716
rat-Cas    ----------------------------K-N-V----G---------------------- 719
murine-Cas ----------------------------R-N-M----G---------------------- 719

human-H-2  HDLANWTPAQPLAPGRTGGLGPSDRQLLLFYLEQCEANLTTLTNAVDAFFTAVATNQPPK 776
rat-Cas    ------------V-------------------------------D---------------- 779
murine-Cas ------------V-------------------------------D---------------- 779

human-H-2  IFVAHSKFVILSAHKLVFIGDTLSRQAKAADVRSQVTHYSNLLCDLLRGIVATTKAAALQ 836
rat-Cas    ------------------------------------K---------------------- 839
murine-Cas ------------------------------------Q---------------------- 839

human-H-2  YPSPSAAQDMVERVKELGHSTQQFRRVLGQLAAA 870
rat-Cas    ----------D---------------------- 874
murine-Cas ----------D---------------------- 874
```

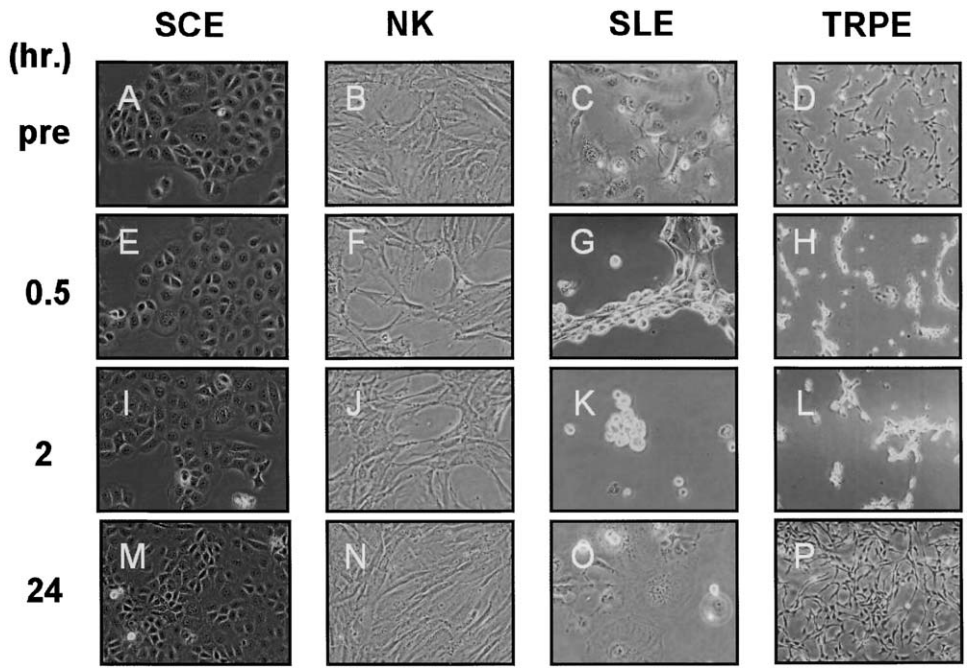


Figure 3. The soluble synthetic tetrapeptides Arg-Gly-Asp-Ser (RGDS) peptides induced only SV40-transformed human lens epithelial cells (SLE) and telomerase reverse transcriptase (TRPE) detachment from plastic culture plates. Cells were seeded on culture plates and cultured for 24 hours. After all cells were confirmed to have adhered to the plates (**A**–**D**), RGDS peptides were added to the culture medium at a final concentration of 1 mM and observed under a microscope for 30 minutes (**E**–**H**), 2 hours (**I**–**L**), and 24 hours (**M**–**P**) thereafter. SLE and TRPE started to detach from the culture plate 30 minutes after the addition of RGDS peptides and were completely detached by 2 hours after peptide addition. However, these cells became reattached to the plate 24 hours later. On the contrary, SV40-transformed human corneal epithelial cells (SCE) and normal human keratocytes (NK) did not show alterations in their morphology throughout the observation period. Similar results were obtained from three separate experiments of identical design.

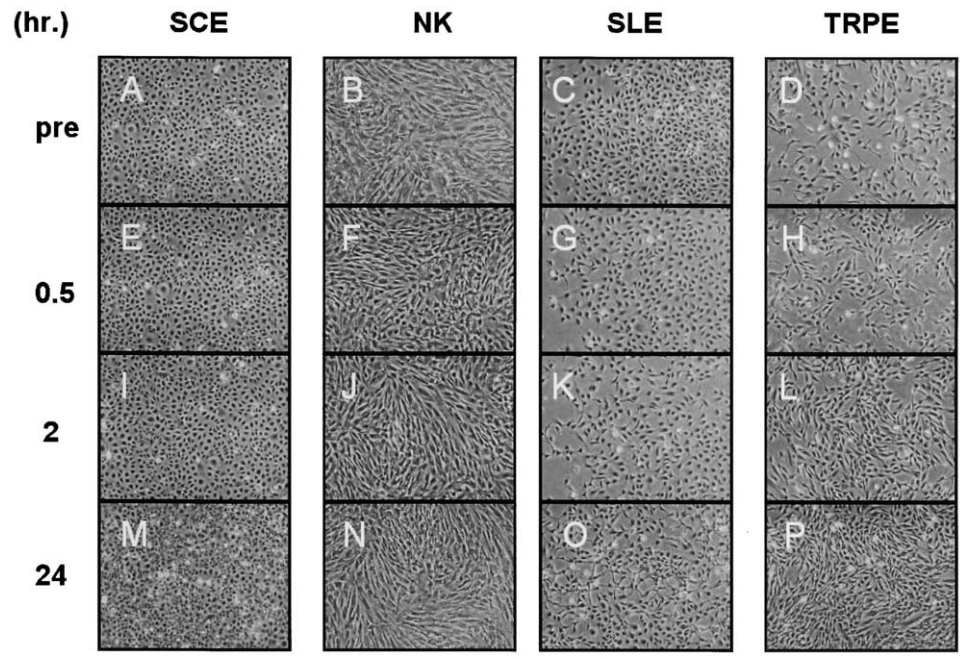


Figure 4. The control peptides Arg-Gly-Glu-Ser (RGES) peptides did not induce detachment of any of the cell types tested from culture plates. After all cells were confirmed to have adhered to the plates (**A**–**D**), RGES peptides were added to the culture medium at a final concentration of 1 mM and observed under a microscope 30 minutes (**E**–**H**), 2 hours (**I**–**L**), and 24 hours (**M**–**P**) thereafter.

Jpn J Ophthalmol
Vol 47: 444–453, 2003

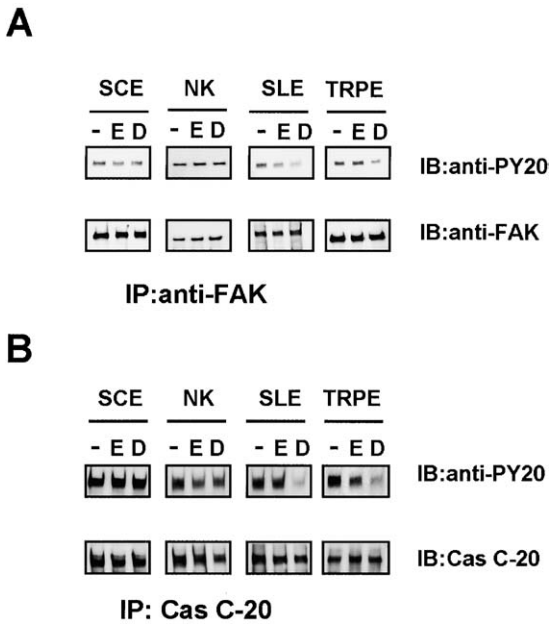


Figure 5. The soluble synthetic tetrapeptides Arg-Gly-Asp-Ser (RGDS) peptides induced a significant decrease in tyrosine phosphorylation of focal adhesion kinase (FAK) and p130 Crk-associated substrate (Cas) on culture plates. Each cell type was seeded on culture plates and treated with RGDS peptides as described in Materials and Methods. (**A**) Cell lysates were immunoprecipitated with anti-FAK and immunoblotted with PY20 or anti-FAK. (**B**) Cell lysates were immunoprecipitated with Cas C-20 and immunoblotted with PY20 or Cas C-20. RGDS peptides induced decreased tyrosine phosphorylation of both FAK and Cas in SV40-transformed human lens epithelial cells (SLE) and telomerase reverse transcriptase (TRPE) but not in SV40-transformed human corneal epithelial cells (SCE) and normal human keratocytes (NK). –: no treatment, E: RGES-treated, D: RGDS-treated.

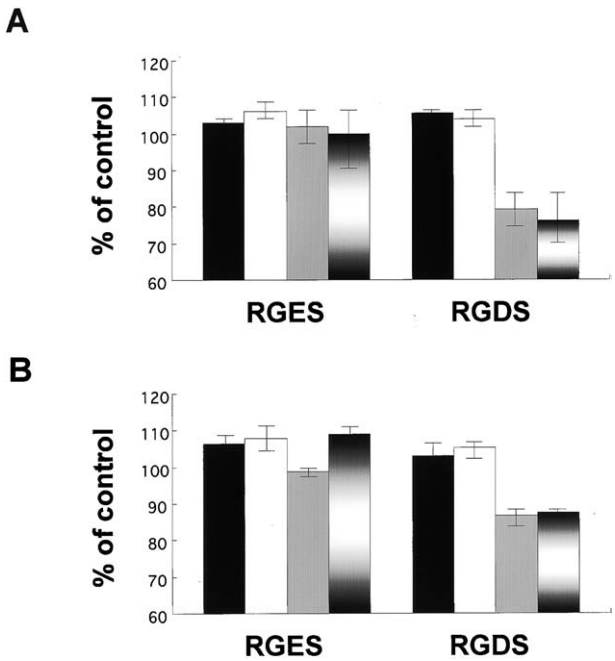


Figure 6. The effects of The soluble synthetic tetrapeptides Arg-Gly-Asp-Ser (RGDS)/control peptides Arg-Gly-Glu-Ser (RGES) peptides on the level of tyrosine phosphorylation of focal adhesion kinase (FAK) and p130 Crk-associated substrate (Cas). The ratio of phosphorylated protein (the band immunoblotted with PY20) and total FAK (Cas) protein [the band immunoblotted with anti-FAK (Cas-C20)] in each experiment with each cell type was evaluated. Then, the ratio of untreated cells was considered as 100% in each cell type, and the percent of the phosphorylated rate of RGDS/RGES-treated cells to untreated cells was calculated as the effect of RGDS/RGES peptides on the level of tyrosine phosphorylation of FAK and Cas. ■: SCE, □: NK, ▩: SLE, ■: TRPE.

SLE and TRPE expressed a large amount of the α3 subunit and, to a lesser extent, the α5 subunit (Figure 7).

Discussion

In the present study, we isolated a human homologue of Cas, H-2. There is extensive sequence homology among human Cas, mouse Cas, and rat Cas. This suggested that this protein Cas was preserved, transcending species.

The H-2 protein that we isolated in this study was identical to the breast cancer anti-estrogen resistance 1 protein (BCAR1) that Brinkman et al recently identified.[23] They suggested that BCAR1 plays a pivotal role in driving breast cancer cell proliferation in the presence of anti-estrogens and may be a novel clinical target. While little information is available regarding the role of Cas in the regulation of human cell proliferation or adhesion, the well-characterized rat and mouse Cas may help us to understand the important role of Cas in human cell proliferation and adhesion.

Rat Cas was originally identified as a protein hyperphosphorylated in cells expressing the transforming gene products v-Crk and v-Src.[25] Subsequent reports have implicated Cas in various processes in different cell types, including cell transformation, integrin signaling, and cell migration and invasion. Recently, numerous studies also indicated that the integrin signal pathway through FAK via Cas played an important role in tumor metastasis.[26,27] Our results showing that the levels of tyrosine phosphorylation in both FAK and Cas were highly correlated with the status of cell adhesion indicated that human Cas, as well as rat and mouse Cas, would be one of the key signaling molecules of integrin–substrate interactions.

Because the RGD motif is an integrin recognition motif found in fibronectin,[11–13] it would be reasonable to speculate that the expression patterns of the integrin subunits

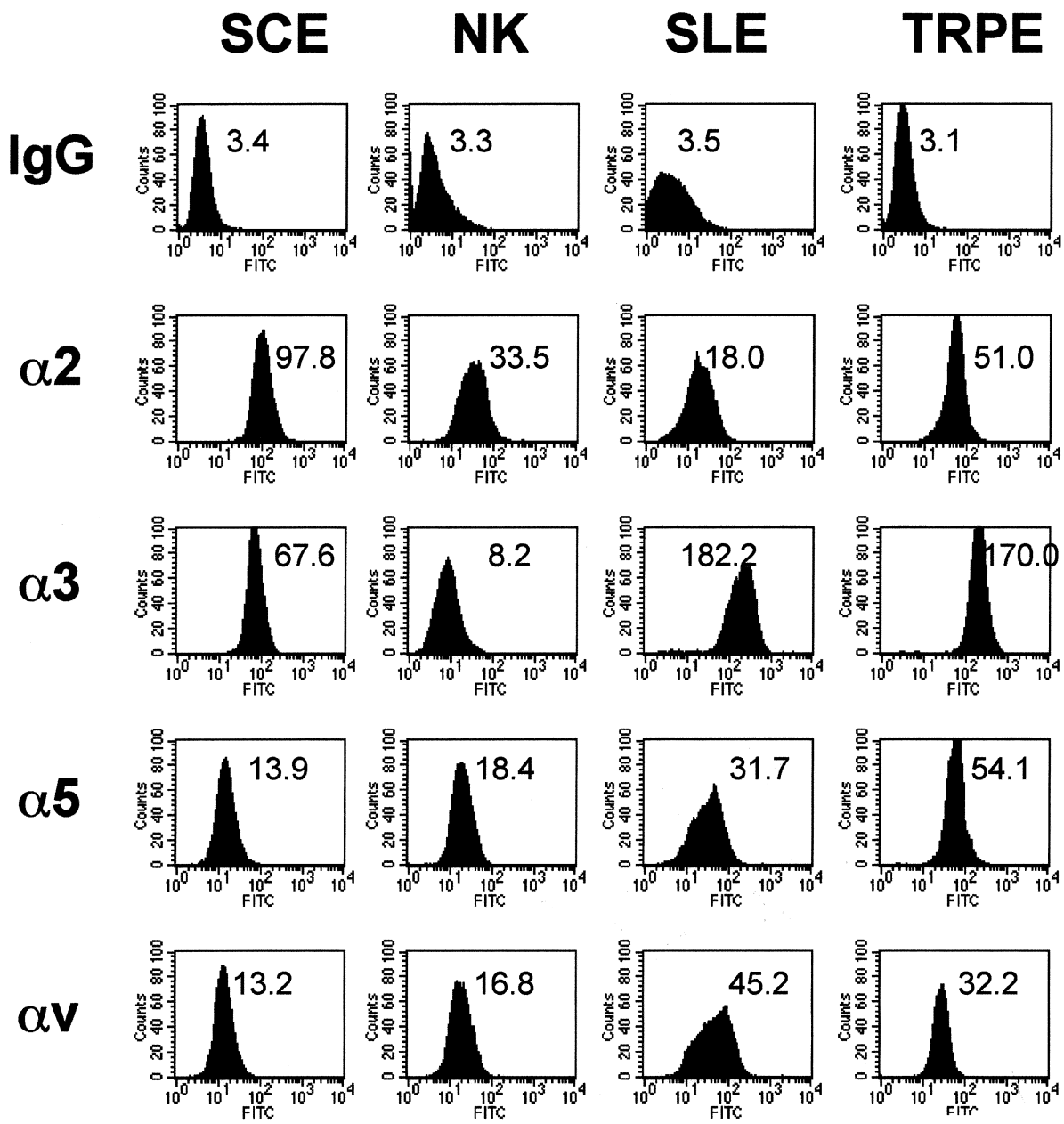


Figure 7. Quantitative analysis of integrin subunits expressed on the cell surface. SV40-transformed human corneal epithelial cells (SCE), normal human keratocytes (NK), SV40-transformed human lens epithelial cells (SLE), and telomerase reverse transcriptase (TRPE) were cultured on culture plates for 24 hours. Cells were then reacted with the anti-integrin α2, α3, α5 or αv antibody, or the isotype control, followed by anti-mouse IgG-FITC. Fluorescence intensities were analyzed using the flow cytometer. α2 and α3 subunits were dominant in SCE, the α2 subunit was dominant in NK, the α3 and α5 subunit were dominant in SLE and TRPE.

caused the different responses to RGD peptides among cells in the present study. Indeed, we found definite differences in the integrin expression patterns among the cells analyzed. Although there is little published quantitative data on the amount of integrin receptors on human corneal epithelial cells, human keratocytes, human lens epithelial cells, and human retinal pigment epithelial cells, previous immunohistological studies have demonstrated that these cell types show different expression patterns of α integrin subunits; normal and tissue-cultured human corneal epithelial cells expressed α2 and α3 integrin,[26] human lens epithelial cells expressed α3 and α6 integrin,[27] and human retinal pigment epithelial cells expressed α3 integrin.[27] In human keratocytes, while

Jpn J Ophthalmol
Vol 47: 444–453, 2003

Lauweryns et al[28] reported that α integrin subunits could not be detected in human corneal keratocytes using immunohistochemistry, Masur et al[29] have reported that αv and α5 integrin were immunoprecipitated in cultured rabbit keratocytes, although they did not investigate the expression of α2 integrin. Concerning keratocytes, the differences between these previous results and ours in this paper are considered to be related to the differences in methods and species. These previous results are consistent with the results reported in this paper.

On plastic culture plates, the matrices to which cells adhere will be mainly the small amounts of vitronectin, fibronectin, or collagen that existed in fetal calf serum or were produced by seeded cells. While collagen also has an RGD sequence, previous studies indicated that α2β1 receptors attach to collagen via non-RGD sequences.[30] On the other hand, the α2β1 integrin dimer recognizes a triple helical structure in the RGD motif of collagen and cyclic RGD peptides have been found to bind to α2β1.[31] Previous studies have also demonstrated that while cyclic RGD peptides inhibited the binding of α2β1 to collagen I, the linear RGDS sequence did not.[32] Therefore, it is reasonable that exogenous linear RGDS peptides, which we used in this study, do not affect SCE and NK expressing high levels of collagen receptor α2β1 integrin.

The detailed mechanism of how RGD peptides forced SLE or TRPE to detach from plastic culture plates still remains unclear. These cells expressed high levels of vitronectin receptor α3β1. Concerning α3β1, while α3β1 has also been implicated in mediating cell adhesion via binding to fibronectin, the RGD sequence is not required for α3β1 integrin-mediated cell adhesion.[33] One possibility is that these cells attached on plastic culture plates mainly via α5β1 integrin, which was also expressed in these cells, although to a lesser extent. When these cells dissociate focal adhesions from the substrate to migrate on plastic culture plates, RGD peptides may inhibit rearrangement of focal adhesions by competition with fibronectin on the plastic culture plates. Thus, both SLE and TRPE may float after the addition of RGD peptides from plastic culture plates. Alternatively, Buckley et al have recently indicated that RGD peptides were able to induce apoptosis directly without any requirement for integrin-mediated cell clustering or signals.[34] The other aspects except integrin expression patterns may be significant factors to explain our results.

The reason why floating SLE and TRPE reattached to the culture plate 24 hours after adding RGDS peptides may be explained by the recent study reported by Buckley et al.[34] They clearly showed that linear RGD-containing peptides entered cells. Therefore, after adding RGDS peptides, the concentration of RGDS peptides in the culture medium will decrease with time, resulting in the reattachment of floating cells. Indeed, Sasabe et al reported that TOTL-86 cells (rabbit lens epithelial cells) also reattached within 144 hours after adding RGDS peptides. Subsequently the addition of RGDS peptides forced these cells to float again.[35]

The results of this study indicated that the integrin signaling pathway through FAK via Cas is essential in cell adhesion to the substrate in eye tissue. Because disturbing this pathway will inhibit cell adhesion to the substrate, this intracellular pathway can be one of the drug-targeting points in eye diseases derived from epithelial cell disorders, such as secondary cataract formation and proliferative vitreoretinopathy.

Supported by Grant-in-Aid C-12671707 for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (AY).

References

1. Honn KV, Tang DG. Adhesion molecules and tumor cell interaction with endothelium and subendothelial matrix. Cancer Metastasis Rev 1992;11:353–375.
2. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992;69:11–25.
3. Hanks SK, Calalb MB, Harper MC, Patel SK. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. Proc Natl Acad Sci USA 1992;89:8487–8491.
4. Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT. pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci USA 1992;89:5192–5196.
5. Guan JL. Focal adhesion kinase in integrin signaling. Matrix Biol 1997;16:195–200.
6. Zachary I, Rozengurt E. Focal adhesion kinase (p125FAK): a point of convergence in the action of neuropeptides, integrins, and oncogenes. Cell 1992;71:891–894.
7. Schlaepfer DD, Hauck CR, Sieg DJ. Signaling through focal adhesion kinase. Prog Biophys Mol Biol 1999;71:435–478.
8. Sakai R, Iwamatsu A, Hirano N, et al. A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J 1994;13: 3748–3756.
9. Burridge K, Turner CE, Romer LH. Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. J Cell Biol 1992;119:893–903.
10. Schlaepfer DD, Hauck CR, Sieg DJ. Signaling through focal adhesion kinase. Prog Biophys Mol Biol 1999;71:435–478.
11. Ruoslahti E. RGD and other recognition sequences for integrins. Annu Rev Cell Dev Biol 1996;12:697–715.
12. Meredith JE, Schwartz MA. Integrins, adhesion and apoptosis. Trends Cell Biol 1997;7:146–150.
13. Werb Z. ECM and cell surface proteolysis: regulating cellular ecology. Cell 1997;91:439–442.
14. Avery RL, Glaser BM. Inhibition of retinal pigment epithelial cell attachment by a synthetic peptide derived from the cell-binding domain of fibronectin. Arch Ophthalmol 1986;104:1220–1222.

15. Nishida T, Nakagawa S, Watanabe K, Yamada KM, Otori T, Berman MB. A peptide from fibronectin cell-binding domain inhibits attachment of epithelial cells. Invest Ophthalmol Vis Sci 1988;29:1820–1825.
16. Nishi O. Posterior capsule opacification. Part 1: Experimental investigations. J Cataract Refract Surg 1999;25:106–117.
17. Ho TC, Del Priore LV. Reattachment of cultured human retinal pigment epithelium to extracellular matrix and human Bruch's membrane. Invest Ophthalmol Vis Sci 1997;38:1110–1118.
18. Yang CH, Huang TF, Liu KR, Chen MS, Hung PT. Inhibition of retinal pigment epithelial cell-induced tractional retinal detachment by disintegrins, a group of Arg-Gly-Asp-containing peptides from viper venom. Invest Ophthalmol Vis Sci 1996;37:843–854.
19. Araki-Sasaki K, Ohashi Y, Sasabe T, Hayashi K, Watanabe H, Tano Y, Handa H. An SV40-immortalized human corneal epithelial cell line and its characterization. Invest Ophthalmol Vis Sci 1995; 36:614–621.
20. Sai T, Sasabe T, Nakamura A. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinase by human corneal keratocytes [ARVO Abstract]. Invest Ophthalmol Vis Sci 2000;41: S698. Abstract nr 3726.
21. Ibaraki N, Chen SC, Lin LR, Okamoto H, Pipas JM, Reddy VN. Human lens epithelial cell line. Exp Eye Res 1998;67:577–585.
22. Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 1984;309:30–33.
23. Kozak M. An analysis of vertebrate mRNA sequences: initiations of translational control. J Cell Biol 1991;115:887–903.
24. Brinkman A, van der Flier S, Kok EM, Dorssers LCJ. BCAR1, a Human Homologue of the adapter protein p130Cas, and antiestrogen resistance in breast cancer cells. J Natl Cancer Inst 2000;92: 112–120.
25. Nojima Y, Miura T, Morino N, et al. Tyrosine phosphorylation of p130Cas in cell adhesion and transformation. Hum Cell 1996;9: 169–174.
26. Tervo K, Tervo T, van Setten GB, Virtanen I. Integrins in human corneal epithelium. Cornea 1991;10:461–465.
27. Menko AS, Philip NJ. Beta 1 integrins in epithelial tissues: a unique distribution in the lens. Exp Cell Res 1995;218:516–521.
28. Lauweryns B, van den Oord JJ, Volpes R, Foets B, Missotten L. Distribution of very late activation integrins in the human cornea. An immunohistochemical study using monoclonal antibodies. Invest Ophthalmol Vis Sci 1991;32:2079–2085.
29. Masur SK, Cheung JKH, Antohi S. Identification of integrins in cultured corneal fibroblasts and in isolated keratocyte. Invest Opthalmol Vis Sci 1993;34:2690–2698.
30. Kamei M, Lewis JM, Hayashi A, Sakagami K, Ohji M, Tano Y. A new wound healing model of retinal pigment epithelial cells in sheet culture. Curr Eye Res 1996;15:714–718.
31. Ruggiero F, Comte J, Cabanas C, Garrone R. Structural requirements for $\alpha 2\beta 1$ and $\alpha 2\beta 1$ integrin mediated cell adhesion to collagen V. J Cell Sci 1996;109:1865–1874.
32. Cardarelli PM, Yamagata S, Taguchi I, Gorcsan F, Chiang SL, Lobl T, Cardarelli PM, Yamagata S, Taguchi I, et al. The collagen receptor $\alpha 2\beta 1$, from MG-63 and HT 1080 cells, interacts with a cyclic RGD peptide. J Biol Chem 1992;267:23159–23164.
33. Wu C, Chung AE, McDonald JA. A novel role for $\alpha 3\beta 1$ integrins in extracellular matrix assembly. J Cell Sci 1996;108:2511–2523.
34. Buckley CD, Pilling D, Henriquez NV, et al. RGD peptides induce apoptosis by direct caspase-3 activation. Nature 1999;397:534–539.
35. Sasabe T, Suwa Y, Kiritoshi A, Doi M, Yuasa T, Kishida K. Differential effects of fibronectin-derived oligopeptides on the attachment of rabbit lens epithelial cells in vitro. Ophthalmic Res 1996;28: 201–208.