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Corneal IL-8 Expression Following Adenovirus Infection Is Mediated by c-Src Activation in Human Corneal Fibroblasts

Kanchana Natarajan,* Maitreyi S. Rajala,* and James Chodosh²*†

Emerging evidence indicates that intracellular signaling cascades mediate entry of pathogenic adenoviruses into target host cells as well as some of the undesirable inflammatory responses to adenoviral gene vectors. We found that Ad19 infection of cultured human corneal fibroblasts induced IL-8 gene transcription independently of IL-1β, TNF-α, and viral gene expression, suggesting that intracellular signaling events might mediate early inflammatory events in adenovirus keratitis. Heat but not UV light inactivation of the virus abrogated the effect of infection on IL-8 mRNA and protein levels, consistent with a viral binding-mediated mechanism. The tyrosine kinase inhibitor herbimycin blocked Ad19-induced IL-8 expression. Western blot analysis revealed tyrosine phosphorylation of the functionally related kinases c-Src and extracellular signal-regulated kinase (ERK) 1/2 in corneal fibroblasts within 15 min after infection. respective inhibitors of these kinases, PP2 and PD98059, also blocked Ad19-induced IL-8 mRNA and protein expression. Application of inhibitors to Src and ERK kinase assays suggested an upstream relationship of c-Src to ERK. Finally, DNA microarray studies performed 1 h after Ad19 or mock infection of corneal fibroblasts in the presence or absence of the Src-specific inhibitor PP2 confirmed a relationship between c-Src and IL-8 expression in Ad19-infected corneal cells. c-Src may act as a global regulator of early proinflammatory host responses to Ad19 infection of the human cornea. The Journal of Immunology, 2003, 170: 6234–6243.

A
denoviruses, nonenveloped viruses with a dsDNA genome, have been implicated in a wide variety of clinical diseases (1, 2). Human adenoviruses are classified into six subgroups (A–F) comprising 51 serotypes. Many adenoviruses infect the eye, but only the group D adenoviruses Ad8, Ad19, and Ad37 cause epidemic keratoconjunctivitis (EKC) (3), the only adenoviral syndrome with significant corneal involvement. EKC is characterized by conjunctivitis and a punctate epithelial keratitis 1 wk to 10 days postexposure, followed by onset of multifocal subepithelial (stromal) corneal infiltrates. These foci of corneal inflammation lead to photophobia and reduced vision for months to years following infection.

In EKC, the multifocal and superficial location of the corneal stromal infiltrates suggests the participation by infected superficial keratocytes in subsequent innate immune responses. Keratocytes, the resident cells of the corneal stroma, maintain the cornea in a precisely organized and transparent state (4, 5). Keratocytes communicate with one another through gap junctions and play an active role in host responses to infection by the production of cyto-
kines and chemokines, including IL-6 (6), RANTES (7), monocyte chemotactant protein-1 (MCP-1) (7), and neutrophil-specific chemokines such as GRO-α (8) and IL-8 (9–12).

Adenovirus binding to target cells is mediated by its penton fiber protein, the major determinant of adenovirus tropism (13–15). Efficient infection of cells by adenoviruses involves an interaction with two cellular receptors (16). First, the fiber knob recognizes and binds to the primary receptor, which in most cases is the coxsackie-adeno-
virus receptor (17–19). Second, an Arg-Gly-Asp (RGD) sequence motif in the viral penton base interacts with the integrins αβ3 or αβ5 (20–22), leading to integrin clustering (focal adhesion formation). This results in recruitment of several signaling molecules to the focal adhesion, including focal adhesion kinase (FAK), Src family kinases, and integrin-linked kinases, followed by integrin-mediated signaling and virus internalization (16, 23, 24).

Our previous studies indicated that cultured corneal keratocytes secrete increased amounts of IL-8 upon infection by the group D adenovirus, Ad19 (9), suggesting a role for IL-8 in adenovirus ocular pathogenesis. We recently demonstrated activation of FAK upon adenovirus infection, and further showed that echistatin, an inhibitor of focal adhesion formation, blocks IL-8 expression by Ad19-infected keratocytes (10), suggesting a link between Ad19-induced expression of IL-8 and intracellular signaling events. In the present study, we demonstrate that increased IL-8 expression upon Ad19 infection of cultured keratocytes follows tyrosine kinase activation within an integrin-dependent signal transduction pathway.

Materials and Methods

Immunologic reagents

HRP-conjugated sheep anti-mouse and donkey anti-rabbit IgG and the chemiluminescent reagents were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The mAb to phosphotyrosine (mAb-P-Y) was obtained from Cell Signaling Technology (Beverly, MA), and the polyclonal ERK 1/2 Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

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1 Abbreviations used in this paper: EKC, epidemic keratoconjunctivitis; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; HCF, human corneal fibroblast; IKK, inhibitory IκB kinase; MAP, mitogen-activated protein; MCP, monocyte chemotactant protein; MEK, MAP/ERK kinase; GC, guanine-cytosine; Tm, melting temperature.

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obtained from Promega (Madison, WI). The c-Src-specific inhibitor PP2, tyrosine phosphorylation inhibitor herbinycin, and mitogen-activated protein (MAP)/ERK kinase (MEK) inhibitor PD98059 were obtained from Calbiochem (San Diego, CA). The partial inhibitor of FAK, echistatin, was obtained from Sigma-Aldrich (St. Louis, MO). Neutralizing Abs to IL-1β and TNF-α were purchased from R&D Systems (Minneapolis, MN). Actinomycin D was purchased from Invivogen (Carlsbad, CA).

Cells and viruses

Primary keratocytes were derived from donor corneas (North Florida Lions Eye Bank, Jacksonville, FL), as previously described (11). Briefly, after mechanical debridement of the corneal epithelium and endothelium, corneas were cut into 2-mm-diameter sections, and each section was placed in individual wells of six-well Falcon tissue culture plates (Fisher Scientific, Pittsburgh, PA) with DMEM, containing 10% heat-inactivated FBS, penicillin G sodium, and streptomycin sulfate. Confluent monolayers were removed before confluence of each cell culture. Cells were grown at 37°C in 5% CO2. Cells from multiple donors were pooled, and the cell monolayers were used at passage number three. Following serial passage in serum-containing medium, keratocytes maintain a fibroblast phenotype (25), and are referred to in the remainder of this work as human corneal fibroblasts (HCF). A fibroblast phenotype was confirmed by immunofluorescent staining using antibodies to vimentin (anti-vimentin) and anti-actin (no reactivity) Abs by methods previously described (9). All experiments were performed with primary HCF at third passage. For inhibitor analysis, HCF were pretreated with actinomycin D (5 μg/ml), herbinycin (1 μM), PP2 (50 μM), or PD98059 (50 μM) for 1 h at 37°C before infection, and echistatin for 1 h before infection at a concentration of 0.2 μM. The cells were exposed to the inhibitors at the same concentrations throughout the infection process. Cell toxicity due to the inhibitors was ruled out by trypan blue exclusion performed on cells treated with inhibitors for the same time at the same concentrations as in our experiments.

Ad19 was cultured directly from a human patient’s cornea and grown in MEM 2% FBS (Life Technologies) at 37°C for 5 days. Heat-inactivated virus was generated by incubating the purified virus have been previously demonstrated (9). Virus was purified in Tris (pH 8.0) buffer that contained 80 mM NaCl, 2 mM MgCl2, and 10% Tris-EDTA (pH 8.0). RNaseIn (Promega) was added to the RNA solution to prevent RNase action. Contaminating DNA was removed by DNase I (Promega) treatment, followed by a phenol/chloroform extraction and subsequent ethanol precipitation of the RNA. The RNA was resuspended at a concentration of 1 mg/ml in diethyl pyrocarbonate-treated water. A spectrophotometric reading at a wavelength of 260 nm was used to determine the concentration of RNA. The quality of each RNA sample was determined by calculating the ratio of OD of each RNA sample at 260:280 nm; a ratio of ~1.8 indicated that samples contained only nondegraded RNA.

**RT-PCR**

For synthesis of cDNAs, 5 μg of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) using an oligo dT primer (Promega) as the primer. The reaction mixture for the reverse-transcription reaction was composed of 1.5 U/ml of RNaseIn, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 500 μM 7 dNTPs, and 10 U/ml of Moloney murine leukemia virus reverse transcriptase. A reaction without reverse transcription, which was comprised of all of the above reactants except for the reverse-transcriptase enzyme, was run to rule out the possibility of amplification of contaminating genomic DNA in the PCR step.

For PCR amplification, optimal primers (Tm ~45–55°C, GC content range 55–60%; primer length 14–24 mer) were designed using the Primer Macintosh software and verified for inter- and intrapimer interactions and self dimerization using the Primer3 and Integrated DNA Technologies (Corval. IA) calculator softwares. Because the EIA sequence for Ad19 is unknown at the present, primers for the EIA 13S product (forward, nt 107–125, 5′-TCTGGCTGTTCATGGC-3′ and reverse, nt 663–682, 5′-CCGGTGTCCTTCTTCTAGGG-3′) were designed using the known Ad9 (group D) adenovirus sequence (26) (GenBank accession number AF099665), to yield a 577-bp product. Primers for IL-8 (forward, nt 489–508, 5′-GGTGTCGTGGTATGTGTTAGGG-3′ and reverse, nt 951–970, 5′-CTTGAGGAAGATGATGGC-3′) were designed based on the GenBank (Unigene Hs.624) human IL-8 complete cDNA sequence and amplified a 481-bp product. GADPH primers were generated from the human GADPH sequence (GenBank accession X01677) and amplified a 165-bp product (forward, nt 76–96, 5′-GTCGAATCAACACGATTCTGCGT-3′ and reverse, nt 226–240, 5′-GAGGTGCATGATGATTGTTG-3′). The IL-1β and TNF-α primers were derived from GenBank sequences (accession numbers 14720674 and 37209, respectively). The IL-1β primers (forward, nt 264–283, 5′-TACCTGAGGACCTTCTTCAGT-3′ and reverse, nt 544–562, 5′-GCCAGGACGTACCAGTAGT-3′) amplified a 299-bp product, while the TNF-α primers (forward, nt 402–421, 5′-CTACTCCATGTTCT-3′ and reverse, nt 585–597, 5′-GAAATGACCTGGCAGAC-3′) amplified a 283-bp product.

A total of 2 μl of the cDNA obtained by reverse transcription was used in the PCR mixture composed of 50 nM Tris-HCl (pH 9.0), 50 mM NaCl, 10 mM MgCl2, 200 μM dNTPs, 20 μg/μl primers, and 1 U of Taq polymerase (Promega). Thin-walled PCR tubes were used for the reactions, and the assay was performed on a programmable thermo-minicycler (MJ Research, Waltham, MA) using one cycle that was comprised of a denaturation step at 96°C for 2 min, followed by 30 cycles of 96°C for 1 min and 68°C for 2 min. The final extension step was conducted at 72°C for 5 min. The amplification products were analyzed by gel electrophoresis in 1% agarose gels, and the sizes of the amplicons were verified by comparing them to the 100-bp DNA ladder (Invitrogen). RNA concentrations of samples were normalized using PCR for the presence of transcripts IL-1β, TNF-α, IL-6, GM-CSF, TGF-β, GAPDH, and IL-8 was performed using the CytoExpress kit from Biosource, according to the manufacturer’s instructions.

**Real-time PCR**

Quantitative real-time PCR analysis was performed using the ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA), according to manufacturer’s instructions. IL-8 primers for real-time PCR (forward (nt 85–102), 5′-AGC TGG CCG TGG CTC TTC-3′ and reverse (nt 152–179), 5′-CTG ACA TCT AAG TTC TTT AGC ACT CCT T-3′), with a 94-bp amplified product, were designed from the GenBank IL-8 sequence (see above) using Primer Express software (PE Applied Biosystems). The cDNA concentrations of samples were normalized using quantification of GAPDH mRNA. Amplification curves were generated by monitoring the fluorescence of SYBR Green I as a measure of incorporation into the amplified product. Samples were then analyzed by comparison of the number of PCR cycles required to reach the midpoint of each amplification curve, or threshold cycle (Ct). Comparison of gene expression between two samples was performed by calculating the n-fold difference in mRNA abundance using the formula \( y = 2^{-Ct} \), where \( C_t \) = Ct of sample 1 – Ct of sample 2. For each gene, a range of concentrations for both the forward and reverse primers allowed us to determine the combination with optimum amplification. Reactions lacking template were used to control for primer-dimer formation. To control for contamination by residual genomic DNA, reverse transcription was omitted from parallel cDNA synthesis reactions.
Adenoviral infection and cellular protein recovery

In light of prior evidence that adenovirus-induced intracellular signaling occurs within several minutes of exposure to virus (27), for most analyses we chilled the cells before infection and maintained them at 4°C for 1 h after infection to allow synchronous onset of intracellular signaling in the presence of adenovirus bound to its host cell receptor. HCF grown to 60% confluence in six-well plates were first washed gently four times with chilled MEM 2% FBS, and then kept at 4°C for 30 min before infection in duplicate with cold Ad19 in MEM 2% FBS at a multiplicity of infection of 50 or cold MEM 2% FBS without virus as a control. After 1 h of viral adsorption at 4°C, HCF were allowed to incubate at 37°C for various times before harvest. Cells were harvested by adding 200 μl of chilled lysis buffer consisting of PBS, 1% Triton X-100, 2 mM EDTA, 0.2 mM sodium orthovanadate, along with protease inhibitors including PMSE (1 mM), pepstatin A (5 μg/ml), leupeptin (10 μg/ml), and aprotinin (10 μg/ml), and incubated at 4°C for 5 min. Cells were scrapped from the plates using a cell scraper, and the cell lysates were collected in microfuge tubes and centrifuged at 13,000 × g for 30 min at 4°C. The supernatants were separated from the lysate pellets, and both were frozen for later use.

**SDS-PAGE and immunoblot analysis**

SDS-PAGE on solubilized proteins was performed using 10% acrylamide gels, following spectrophotometric bicinchoninic acid analysis to normalize the loading. Solubilized proteins were mixed with 3% sample buffer (60 mM Tris-HCl (pH 6.8), 10% glycerol, 0.02% (w/v) SDS, 0.05% bromophenol blue (w/v), 2% ME), boiled for 4 min, and immediately loaded on separating gels (10% resolving gel, 4% stacking gel). After SDS-PAGE, gels containing solubilized proteins from lysate supernatants were transferred into nitrocellulose using a Bio-Rad ( Hercules, CA) MiniProtein II transfer apparatus. Nitrocellulose sheets were blocked overnight at 4°C with TBS (10 mM Tris-HCl, pH 7.4, and 300 mM NaCl containing 0.1% Tween 20) containing 5% crystalline grade BSA. Incubations with primary antisera (diluted in blocking buffer) were performed for 2 h at room temperature. Immunoblots were washed three times with TBS after both the primary and secondary Ab incubations. Ab reactivity was determined with ECL reagents using peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit secondary Abs. Before reprobe of developed blots, blots were incubated in 62.5 mM Tris solution (pH 6.5) containing 2% SDS and 0.1 M 2-ME at 50°C for 30 min. Densitometric scans of immunoblots were analyzed by ONE-DS-CAN software (Scanalytics, Bellerica, MA) in the linear range of detection, and absolute values were then normalized. Densitometry parameters included threshold and bandwidth, and were set identically for tested bands.

**Immunoprecipitation and kinase assays**

Cells were solubilized by suspension in 200 μl of ice-cold lysis buffer (PBS pH 7.4, containing 1% Triton X-100, 2 mM EDTA, and 0.2 mM orthovanadate) for 5 min, followed by centrifugation at 13,000 × g for 30 min at 4°C. The supernatants were removed and precleared by mixing with 50 μl protein A agarose for 30 min with gentle mixing at 4°C, followed by centrifugation at 13,000 × g. The cleared supernatants were mixed with 5 μg of anti-ERK-P-Y or anti-Src and 50 μl protein A agarose in lysis buffer and maintained overnight at 4°C. The following morning, the mixture was centrifuged at 6000 × g for 5 min, the supernatant was removed, and the immunoprecipitates were washed thrice with 300 μl of lysis buffer, followed by three washes with kinase assay buffer. The immunoprecipitates were solubilized in kinase assay buffer for further use.

ERK 1/2 kinase assays were performed using the ERK kinase nonradioactive assay kit (Cell Signaling Technology), as per the manufacturer’s instructions. Briefly, phosphorylated ERK 1/2 was immunoprecipitated from 200 μg of total Triton-X-soluble protein sample, resuspended in kinase buffer (Cell Signaling Technology), and incubated in the presence of a GST-Elk fusion protein at 30°C for 30 min. The products of the kinase reaction were then run on an SDS-PAGE gel and transferred onto nitrocellulose membranes. The membrane was then immunoblotted with a phospho-ERK Ab supplied in the kit, and the immunoreaction was detected with ECL detection reagents, as described above. The Src kinase assay was done using the Src kinase assay kit (Upstate Biotechnology, Lake Placid, NY). Steps followed were similar to those in the ERK kinase assay. [γ-32P]ATP was used to phosphorylate the Src substrate peptide in the kinase reaction, and the reaction counts were measured using a scintillation counter. Src activity (picomols of [γ-32P]phosphate incorporated in the substrate peptide per minute) was calculated based on individual reactivity counts as compared with reactivity counts of 20 U of recombinant Src enzyme (Upstate Biotechnology), the specific radioactivity of the [γ-32P]ATP, and the length of incubation, as per manufacturer’s instructions.

**ELISA**

HCF were infected in 48-well tissue culture plates with purified Ad19 or mock infected with OptiMem (Life Technologies) as a control. Cell supernatants were harvested 4 h postinfection, and the amount of IL-8 protein was quantified using a calorimetric sandwich ELISA Quantikine kit from R&D Systems. Plates were read on an Emax microplate reader ( Molecular Devices, Sunnyvale, CA) and analyzed with SOFTmax analysis software ( Molecular Devices). The means of triplicate ELISA values for each of the virus- or mock-infected wells were compared by Student’s t test. A value of p ≤ 0.01 was considered significant.

**Microarray analysis**

HCF were infected with Ad19, as described in the RNA isolation section. Total RNA was isolated from HCF at 1 h postinfection, as described. RNA quality was confirmed, as above. A custom gene profiling analysis was performed by Clontech Laboratories (Palo Alto, CA), using the broad coverage Atlas human 1.2K glass cDNA expression array, which profiles many genes involved in crucial cellular functions, including oncogenes, transcription factors, growth factors and receptors, cytokines and chemokines, and apoptosis, signaling-, metabolism- and cell cycle-related genes. The quality of our RNA was tested by us and then confirmed independently by Clontech. A total of 1 μg of the total RNA from infected and uninfected cells was reverse transcribed using a cDNA synthesis primer mix containing [α-32P]dATP to synthesize probe mixtures. Each radioactive labeled probe mixture was then hybridized to separate Atlas human 1.2K arrays subjected to high stringency washes, and then analyzed by autoradiography and quantified by phosphor imaging. Densitometric analysis was used to standardize the expression of individual genes to a panel of housekeeping controls, and differential gene expression was defined by Clontech at a signal ratio of at least 3:1. The microarray experiment was performed twice with similar results.

**Results**

Early IL-8 gene expression in adenovirus-infected HCF occurs independently of viral replication and is inhibited by the tyrosine kinase inhibitor herbimycin

Previous work in our lab had shown that infection of HCF by Ad19 induced the secretion of IL-8 protein (9). To determine whether Ad19 infection regulates IL-8 gene expression at a transcriptional level, an RT-PCR analysis was done at various times postinfection. At 1 h postinfection, IL-8 message increased in the Ad19-infected sample as compared with the mock-infected sample, while at 45 min, no significant difference in IL-8 gene expression between virus- or mock-infected wells were compared by Student’s t test. The microarray experiment was performed twice with similar results.

We then wished to test whether the induction of IL-8 gene expression upon Ad19 infection could be due to the action of viral gene products. E1A is the earliest gene transcribed in adenovirus replication (28). The primary E1A transcript undergoes differential splicing to yield five distinct messages (29, 30). One E1A message with a sedimentation coefficient of 135 (E1A-13S) is particularly abundant at early times during infection (29). Hence, we sought to determine at what time postinfection the E1A-13S message was evident (Fig. 1a). Equal amplification of the housekeeping gene GAPDH suggested equal loading of cDNA in the duplex PCR. Reverse-transcriptase negative reactions appropriately failed to amplify a product.

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Because IL-1β/H9252, or a CytoXpress multiplex amplification kit (Fig. 1A), samples by herbimycin did not induce higher expression of TNF-α. IL-8 was the only transcript studied to be consistently up-regulated. In other independent experiments, IL-8 mRNA synthesis, but that expression of IL-1β/H9252 had shown no increase in IL-1β and TNF-α transcripts at 1 h postinfection, when IL-8 mRNA was already up-regulated in virus-infected cells (Fig. 1c). To confirm that Ad19 infection of HCF did not induce the expression of these proinflammatory cytokines before the induction of IL-8, we performed an RT-PCR analysis at 1 and 2 h postinfection, using amplification of GAPDH to internally control for equal loading of samples. IL-1β/H9252 transcription was not altered in Ad19-infected cells 1 h after infection, but was up-regulated 2 h postinfection (Fig. 2a). TNF-α gene expression was not altered 1 or 2 h postinfection (data not shown).

To further examine whether IL-1β/H9252 might be the cause of IL-8 expression by Ad19-infected HCF, we treated Ad19-infected HCF with an anti-IL-1β/H9252 Ab at concentrations significantly higher than neutralizing doses. Ad19 infection significantly increased IL-8 levels in HCF supernatants as compared with mock-infected cells (1226 ± 104.2 vs 228 ± 44.1 pg/ml, p ≤ 0.00005, Fig. 2b). Anti-IL-1β/H9252 Ab maximally reduced the secretion of IL-8 at 4 h postinfection by ~20% (896.9 ± 28.2 pg/ml, p ≤ 0.003, Fig. 2b), suggesting that IL-1β/H9252 expression might augment IL-8 protein synthesis, but that expression of IL-1β/H9252 is not required for IL-8 production by Ad19-infected HCF. Greater concentrations of anti-IL-1β/H9252 Ab resulted in no additional reduction in IL-8 protein levels (data not shown). The addition of an anti-TNF-α/H9252 Ab to cells already exposed to the anti-IL-1β/H9252 Ab induced no additional reduction in IL-8 protein at 4 h postinfection (Fig. 2b), consistent with our RT-PCR data for TNF-α/H9252.

In separate experiments, heat-inactivated Ad19 failed to induce secretion of IL-8 protein much beyond basal levels, while UV inactivation of the virus induced IL-8 levels close to those induced by untreated Ad19 and significantly greater than those in mock-infected cells (p ≤ 0.02 and 0.00009, respectively, Fig. 2c), indicating that viral replication was not, but viral binding to its host receptor was necessary for IL-8 expression. Taken together, these data suggest that IL-8 expression is up-regulated initially as a consequence of Ad19 binding to HCF, and may be subsequently enhanced by an indirect effect of the proinflammatory cytokine IL-1β/H9252.

**Ad19 infection induces activation of c-Src and ERK 1/2 in HCF**

Group C adenovirus infection of immortalized epithelial cell lines has been shown to induce the phosphorylation of several signaling proteins, including FAK, c-Src, phosphoinositide-3 kinase, Ras, Raf, and MEK 1/2 (16, 27). To determine whether the interaction of Ad19, a group D adenovirus, with HCF also activates a cellular signaling cascade, Triton-X-soluble proteins from Ad19- and mock-infected cells at different times postinfection were run on SDS-PAGE gels and immunoblotted for phosphotyrosine residues. Tyrosine-phosphorylated proteins of ~60 and 40 kDa molecular mass were consistently noted in repeated experiments in the Ad19-infected samples at 15 min postinfection (Fig. 3a). Parallel experiments performed at 37°C demonstrated phosphorylation of the these two cytokines preceded IL-8 gene transcription. Earlier data had shown no increase in IL-1β/H9252 and TNF-α/H9252 transcripts at 1 h postinfection, when IL-8 mRNA was already up-regulated in virus-infected cells (Fig. 1c). To confirm that Ad19 infection of HCF did not induce the expression of these proinflammatory cytokines before the induction of IL-8, we performed an RT-PCR analysis at 1 and 2 h postinfection, using amplification of GAPDH to internally control for equal loading of samples. IL-1β/H9252 transcription was not altered in Ad19-infected cells 1 h after infection, but was up-regulated 2 h postinfection (Fig. 2a). TNF-α gene expression was not altered 1 or 2 h postinfection (data not shown).

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Table I. Effect of actinomycin D on IL-8 mRNA levels by real-time PCR

<table>
<thead>
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Because IL-1β/H9252 and TNF-α/H9252 are known to up-regulate IL-8 gene expression, we sought to determine whether expression of either of
samples that were significantly different from controls. Media vs Ad19 (p ≤ 0.00005); Ad19 vs Ad19 + anti-IL-1β (p ≤ 0.003) (C) HCF were either mock infected or infected with wild-type (wt), heat- or UV-inactivated Ad19 for 4 h at 37°C before collecting the cell supernatants for quantification of IL-8 protein expression by ELISA. Media vs Ad19 (p ≤ 0.00005); media vs UV-inactivated Ad19 (p ≤ 0.000009) (wt Ad19 vs UV-inactivated Ad19 (p ≤ 0.02)). The PCR experiments were performed five times each, and the ELISAs are representative of three independent experiments with similar results.

**FIGURE 3.** Src and ERK 1/2 are phosphorylated in response to Ad19 infection in HCF. Cell cultures were infected with Ad19 or mock infected with virus-free medium, and after 1 h of adsorption at 4°C were incubated at 37°C for 15 min. The cells were solubilized, and the solubilized proteins were subjected to SDS-PAGE and immunoblotted with mAb-P-Y (A); immunoblotted with anti-phospho-Src Ab (src-P-Y419), stripped, washed, and reprobed with anti-Src (B); or immunoblotted with anti-phospho-ERK 1/2 Ab (ERK 1/2-P-T202/P-Y204), stripped, washed, and reprobed with ERK 1/2 (C). Densitometric values shown are normalized to compensate for any changes in Src or ERK 1/2 in the solubilized protein component. These experiments were performed three times with similar results.
and selective inhibitor of Src kinase family members (39). To confirm that PP2 would inhibit c-Src activity in HCF, a Src kinase assay was done in the presence or absence of a previously published concentration of PP2 (50 μM) in mock- and Ad19-infected HCF. Echistatin, a snake venom disintegrin that partially inhibits FAK, served as a negative control inhibitor. Notably, neither PP2 nor echistatin altered total ERK levels in uninfected cells (data not shown). As seen in Fig. 4a, PP2 (50 μM) reduced Ad19-induced Src kinase activity ~180-fold at 5 min postinfection. Levels of phosphorylation of ERK 1/2 were reduced in Ad19-infected HCF in the presence of 50 μM PP2 (Fig. 4b), suggesting that ERK 1/2 was downstream of c-Src. Echistatin (0.2 μM), the highest concentration that did not induce cell toxicity in HCF (data not shown), appeared to have no significant effect on ERK 1/2 phosphorylation. A serine residue within Elk-1 acts as a substrate for phosphorylation by ERK 1 and ERK 2 (42–44). PP2 reduced phosphorylation of Elk-1 fusion protein in the ERK kinase assay (Fig. 4c), indicating the importance of c-Src function to downstream ERK activation. PD098059 (50 μM), a known inhibitor of ERK 1/2 kinase activity (35, 36) and a positive control inhibitor in the ERK kinase assay, reduced levels of Elk-1 fusion protein phosphorylation, while echistatin had no significant effect (Fig. 4c).

**Ad19 infection-induced IL-8 expression is reduced by specific inhibitors of c-Src and ERK 1/2**

To determine whether the identified signaling proteins play a role in IL-8 expression, RT-PCR analysis was done to compare levels of IL-8 message in Ad19-infected cells as compared with mock-infected HCF in the presence or absence of specific inhibitors of Src and ERK activity. The tyrosine kinase inhibitor herbimycin and the c-Src inhibitor PP2 each reduced levels of IL-8 message, suggesting that tyrosine phosphorylation and c-Src activation and activity are required for Ad19-induced transcription of IL-8 (Fig. 5a).

ERK 1/2 is known to be downstream of MEK 1 (35, 36). We applied a MEK 1 inhibitor, PD098059, to examine the effect of inhibition of ERK activation on IL-8 expression. PD098059 inhibited transcription of IL-8 at 1 h postinfection (Fig. 5a), suggesting a role for ERK 1/2 in IL-8 gene expression. At the protein level, all three signaling inhibitors, herbimycin, PP2, and PD098059, significantly reduced IL-8 protein secretion ($p \leq 0.00004, 0.00004$, and 0.00009, respectively) in Ad19-infected HCF to uninfected levels (Fig. 5b). No toxicity was observed with any of these chemicals by trypan blue exclusion. These data suggest that c-Src and ERK 1/2 activation are necessary for early IL-8 gene transcription and protein synthesis in Ad19-infected HCF.

**Inhibition of c-Src activity blocks IL-8 gene expression without compensatory increases in other proinflammatory cytokines**

Microarray-based gene expression analysis was performed to determine the effect of c-Src inhibition on cellular gene expression in mock- and Ad19-infected HCF. The transcription of only a few genes of the 1176 tested increased greater than 3-fold at 1 h postinfection (Fig. 6). Interestingly, MCP-1 and ICAM-1 were up-regulated in Ad19-infected HCF as compared with mock-infected cells. However, of the genes on the array that showed an increase in transcription in response to Ad19 infection, IL-8 was up-regulated to the greatest degree (relative 5-fold increase in infected HCF as compared with mock-infected cells), confirming our earlier data and the primacy of this chemokine in the early inflammatory response to corneal stromal infection with Ad19. The microarray analysis additionally showed that inhibition of IL-8 gene
transcription with PP2 also blocked MCP-1 and ICAM-1 transcription, but did not induce any compensatory increases in other proinflammatory cytokines (Fig. 6).

Discussion

Epidemic keratoconjunctivitis is the only adenoviral eye disease with significant corneal involvement. Neutrophils are the first infiltrating cells in the cornea of experimental animals with adenovirus keratitis (45), implicating the presence of neutrophil chemokines in the corneal stroma as an early proinflammatory signal (9).

General mechanisms that can lead to increased IL-8 gene expression and secretion in virus-infected cells include oxidative stress (46), viral gene product activation of host gene transcription (47, 48), and protein kinase signaling cascades initiated by viral binding and internalization (27, 49). We present evidence that binding of Ad19 (one of the subgroup D adenovirus serotypes that causes EKC) to HCF in vitro induces an intracellular signaling cascade involving e-Src and the MAP kinases ERK 1/2. One of the earliest downstream effects of this cascade appears to be the secretion of the proinflammatory chemokine, IL-8, possibly the key chemotactic signal for the migration of the neutrophils into the corneal stroma in EKC (9).

The ability of UV-inactivated viruses to induce cytokines has previously been shown in a variety of systems (50, 51), indicating that virus replication is not always necessary to initiate a host response. The absence of one of the most abundantly expressed early adenoviral gene transcripts, E1A-13S, at a time when IL-8 message is already up-regulated, and the ability of HCF to secrete IL-8 when infected with UV-inactivated Ad19 indicate that Ad19 replication is not essential to early IL-8 production. Heat inactivation of the virus theoretically interferes with its binding to the target cell. Heat-inactivated Ad19 failed to induce IL-8 expression in HCF, and taken together with our finding that actinomycin D also abrogates Ad19-induced IL-8 expression, suggests a possible effect of Ad19 binding on IL-8 transcription. The apparent effect of actinomycin D on IL-8 mRNA levels might also be due to reduced synthesis of other proteins that stabilize mRNA transcripts. Therefore, determination of the effect of Ad19 infection on IL-8 transcriptional activation clearly awaits more detailed studies. Our PCR and microarray analyses do suggest that early IL-8 production does not require other proinflammatory cytokines such as TNF-α or IL-1β. Our ELISA data indicate that IL-1β may contribute to IL-8 protein secretion later on in infection. When viewed holistically, these data suggest that the initial increase in IL-8 protein may be a direct effect of Ad19 binding on IL-8 transcription, and that IL-8 protein expression may be subsequently enhanced by the action of the proinflammatory cytokine IL-1β.

Adenoviruses use two cellular components to interact with and enter susceptible cells. The primary interaction occurs between the adenovirus fiber knob and a cell surface molecule such as coxsackie-adenovirus receptor, followed by a secondary interaction between the RGD domain on the adenovirus penton base and αv integrins on the cell surface (16, 52). The second interaction leads
to integrin activation and is known to induce a phosphoinositol-3 kinase-dependent pathway within the cells, leading to internalization of the virus (53). In addition to this pathway, other pathways may be initiated by integrin activation (23, 54). For example, activation of ERK 1/2 in a transformed epithelial cell line by adenovirus type 7 has been previously shown (55). In turn, activation of ERK 1/2 can trans-activate the IL-8 transcription factors NF-κB and AP-1 (56), and has been shown to induce IL-8 expression in several different experimental systems (55–58). We suggest that Ad19 binding to HCF induces a pathway including the tyrosine kinases c-Src and ERK 1/2. Based on inhibitor analyses of phosphorylation and activity, we further suggest that c-Src is linearly upstream of ERK 1/2, and that both of these proteins play a role in the inflammatory response to infection, as their activation is necessary for IL-8 expression by Ad19-infected HCF (Fig. 7).

As a result of integrin clustering via the binding of a multivalent ligand, FAK, a nonreceptor protein tyrosine kinase localized at focal adhesions, is autophosphorylated at its tyrosine residue 397, and this creates a high affinity binding site for the Src homology 2 domains of Src family kinases (59). This interaction between FAK and Src kinases leads to the subsequent activation of downstream MAP kinases that in turn mediate cellular cytokine gene transcription (60). Earlier studies in our lab have shown that Ad19 infection of HCF induces FAK autophosphorylation at FAK-PY397 (10), possibly leading to IL-8 expression. Our data presented in this work indicate that inhibition of phosphorylation at FAK-PY397 does not alter activity of c-Src or ERK 1/2. Possibly, FAK and Src activation occur as part of two independent signaling events initiated by Ad19-mediated integrin activation at the surface of HCF, and these independent pathways either converge at some point downstream of ERK 1/2, or independently lead to IL-8 expression.

DNA microarray technology can be used to discover what genes are expressed in a particular disease state. Host responses to various pathogens, including bacteria and viruses, have been studied using DNA microarrays (61–69), and in a few instances, IL-8 was one of the inflammatory mediators up-regulated as a consequence of infection (67–69). Our microarray analysis of cDNA from mock- and Ad19-infected HCF at 1 h postinfection revealed that IL-8 gene expression was induced to a greater degree than any of the other 1176 genes tested. The absence of expression at 1 h postinfection of other proinflammatory cytokines, such as TNF-α and IL-1β, that could potentially up-regulate IL-8, was also consistent with IL-8 production as a consequence of a signaling cascade initiated by Ad19 binding to HCF.

We have not explored the time course of IL-8 expression in Ad19-infected HCF. Later events, including possible down-regulatory influences of putative factors such as viral gene products or host cell-derived cytokines, are unlikely to have much effect on neutrophil infiltration into the cornea, because IL-8, once bound by negatively charged proteins within extracellular matrix, is exceptionally stable (70). Once IL-8 has been secreted into the corneal stroma, the possible late inhibition of IL-8 expression becomes irrelevant; the signal for neutrophil infiltration once present in the stroma cannot be taken back. Therefore, early IL-8 expression by Ad19-infected keratocytes would most likely initiate chemotaxis and infiltration by neutrophils regardless of later events.

By DNA array analysis, we also observed an up-regulation of other inflammatory mediators and transcription factors in response to Ad19 infection. Of those genes that were differentially regulated, ICAM-1 and MCP-1 are interesting candidates for further study, as these molecules may act as part of an inflammatory host response to infection (71–73). ICAM-1 is an inducible cell adhesion glycoprotein of the Ig supergene family expressed on the surface of a wide variety of cell types (74), and may be up-regulated in response to proinflammatory cytokines, hormones, cellular stresses, and virus infection to promote intercellular signaling during inflammation (75). MCP-1, a member of the C-C chemokine family, is chemotactic for monocytes and other leukocyte subsets (76). The promoters of the IL-8, MCP-1, and ICAM-1 genes all contain binding sites for and are regulated by the transcription factors AP-1 and NF-κB (77, 78), and the IKK/IκB/NF-κB pathway has been shown to be important for the up-regulation of ICAM-1, MCP-1, and IL-8 in endothelium (79). Thus, different stimuli can activate similar transcription factors that in turn mediate simultaneous expression of ICAM-1, MCP-1, and IL-8 (80, 81). Interestingly, all the genes up-regulated by Ad19 infection on microarray analysis were inhibited by PP2, suggesting a global role for c-Src in regulation of the early host responses to Ad19 infection. Also, as signaling pathways induced by the virus may play a role in adenovirus ocular pathogenesis, signaling inhibitors such as PP2 may have potential as therapy. Notably, PP2 did not induce compensatory cytokine production by the corneal cells.

Further studies to elucidate the molecular mechanisms by which group D adenoviruses induce corneal inflammation may lead to the development of new and novel therapeutic agents against EKC.

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References


