Expression of the Gene Encoding Poly(ADP-ribose) Polymerase-1 Is Modulated by Fibronectin during Corneal Wound Healing

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PURPOSE. Poly(ADP-ribose) polymerase (PARP)-1 is a nuclear enzyme essential in several cellular functions such as DNA repair, DNA transcription, carcinogenesis, and apoptosis. Expression of the PARP-1 gene is mainly dictated by the transcription factor Sp1. Fibronectin (FN), a component from the extracellular matrix transiently expressed at high levels during wound healing of the corneal epithelium, was reported to exert a positive influence on expression of the α5 integrin subunit gene promoter by altering the state of Sp1 phosphorylation, a process that depended on the activation of the ERK signalling pathway. The present study was undertaken to investigate whether PARP-1 gene expression might be similarly regulated by FN through the same signalling pathways and attempted to link expression of this gene to wound healing in vitro.

METHODS. Expression of PARP-1, Sp1/Sp3, ERK1/2, phospho-ERK1/2, P38 and phospho-P38 was monitored by Western blot in cultures of rabbit corneal epithelial cells (RCECs) grown on FN in the presence of inhibitors of the MAPK, PI3K, and P38 signalling pathways. Electrophoretic mobility shift assays (EMSAs) were conducted to assess the binding of Sp1 and Sp3 in nuclear extracts from RCECs grown on FN in the presence of inhibitors. Plasmids bearing the PARP-1 promoter fused to the CAT reporter gene were also transfected into RCECs grown under similar culture conditions to assess the influence of these inhibitors on PARP-1 promoter activity.

RESULTS. Expression of PARP-1, Sp1, and Sp3 increased considerably in RCECs grown on FN and translated into increased binding of Sp1 and Sp3 to their DNA target sites. In addition, FN increased PARP-1 promoter activity in a cell-density-dependent manner. Inhibition of both the MAPK and the PI3K pathways entirely abolished these properties.

CONCLUSIONS. PARP-1 gene expression was strongly activated by FN through alterations in the phosphorylation state of Sp1 and Sp3 that resulted from the activation of the MAPK and PI3K signalling pathways, thereby suggesting that PARP-1 may play a critical function during the highly proliferative phase that characterizes wound healing of the corneal epithelium.

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the MAPK pathway, ERK1/2 kinases also appear to be downstream targets of the PI3K but not the p38 pathways. Sp1 can therefore become hyperphosphorylated on activation of either pathway (MAPK and PI3K). Sp1 is the founding member of a Zn-finger family of transcription factors, the Sp family, that now comprises nine Sp genes (Sp1 to Sp9) (reviewed in Ref. 24). Because it is ubiquitously expressed and its GC-rich target site is found in a large number of eukaryotic genes, Sp1 is believed to control and regulate the expression of many thousands genes in the human genome, several of which encoding proteins required for cell maintenance and survival functions such as proliferation, differentiation, metabolism, and apoptosis. A role for Sp1 in corneal wound healing has been postulated, as this transcription factor regulates the expression of many integrin subunit genes at the transcriptional level (which comprises integrin subunits α2, α6, α11, the leukocyte integrin subunits CD11c and CD11d, β2/CD18, αIIb, αv, β5, and β3, as well as the α5 FN-binding integrin subunit). In addition, actively growing, undifferentiated primary cultured cells that are typically found in healing tissue were reported to express high levels of Sp1, whereas quiescent or fully differentiated cells did not.

Beside integrin genes, Sp1 is also believed to regulate the expression of most, if not all, housekeeping genes. One such candidate is the gene encoding poly(ADP-ribose) polymerase (PARP)-1. PARP-1 is a nuclear enzyme that is involved, by posttranslational modification of various proteins, in several important cellular functions, including DNA damage signaling, DNA repair, DNA transcription, carcinogenesis, and apoptosis (for review, see Ref. 39). The transcriptional activity directed by this housekeeping gene promoter is deeply regulated by the transcription factors Sp1/Sp3. As for Sp1, PARP-1 expression and activity appear to be modulated by cell density and differentiation during corneal wound healing. Besides, PARP-1 is often found in active regions of chromatin, most likely because of its role in poly(ADP-ribosyl)ation of histones (for a review see Ref. 42). PARP-1 expression has been postulated to play a protective function during the proliferative phase that characterizes corneal wound healing. Through its action on histone proteins, PARP may also facilitate expression of genes whose products are required for cell adhesion and migration of the leading edge by promoting unwinding of active chromatin. Furthermore, PARP-1 has been recently shown to regulate the expression of the integrin CD11a through direct interaction with NF-κB, establishing a role for PARP-1 in cell migration during neuronal injury. As cell migration is a major prerequisite for wound healing, it is likely that PARP-1 gene expression will be differently modulated during this process.

In this study, we examined whether expression of PARP-1 might be under the regulatory influence of the ECM components FN, LM, and CIV in RCECs to establish a putative function for PARP-1 in corneal wound healing. Inhibitors of the MAPK, PI3K, and p38 signal transduction pathways were used to decipher which of these routes are activated by the ECM. Our data indicate that only FN could increase the activity directed by the rPARP-1 promoter in an integrin-specific and a cell-density–dependent manner. This regulatory influence of FN is mediated by either the MAPK or PI3K pathways as both PD98059 and wortmannin, but not SB203580, could prevent activation of ERK1/2 and thereby alter the phosphorylation state of Sp1 and its regulatory influence on the rPARP-1 promoter activity. The coordinated expression of FN, Sp1, and PARP-1 in highly migrating and proliferative RCECs suggests that PARP-1 must play a critical role during the proliferative burst that characterizes wound healing of the corneal epithelium.

**Materials and Methods**

All experiments described in this article were conducted in voluntary compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures were approved by the Laval University Animal Care and Use Committee.

**Cell Culture and Media**

RCECs were obtained from the central area of freshly dissected rabbit cornea as described previously and then grown to confluence (near 80% coverage of the culture plates) under 5% CO₂ in supplemented corneal epithelial medium (SCEM) with 5% FBS and 20 µg/mL gentamycin. When indicated, human FN, murine laminin type 1 (LM), or human collagen type IV (CIV; all from Sigma-Aldrich, Oakville, ON, Canada), was coated on the culture dishes at varying concentrations (FN, 5 µg/cm²; LM, 2 µg/cm²; CIV, 3 µg/cm²), as described previously. Inhibition of the intracellular signaling pathways was performed by culturing subconfluent RCECs in the presence of 20 µM of the MEK/kinase inhibitor PD98059 (Cell Signaling Technology, Inc. Pickering, ON, Canada), 0.1 µM of the PI3K inhibitor wortmannin (Sigma-Aldrich), or 10 µM of the P38 inhibitor SB203580 (Sigma-Aldrich) for 48 hours before the cells were harvested.

**Plasmids and Oligonucleotides**

The rPARP-1 recombinant plasmids PCR3 and PCR3/F2F3F4m have been described elsewhere. The PXC-GHS plasmid, which bears a secreted version of the human growth hormone gene upstream of the mMT-I promoter, was the kind gift of David D. Moore (Department of Molecular and Cell Biology, Baylor College of Medicine, Houston, TX). The plasmid pCMV-Flag-P38(AGF) which encodes high levels of a dominant negative form of p38 was kindly donated by Roger J. Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA). The double-stranded oligonucleotides bearing the high-affinity binding site for either Sp1 (5'-GATCATATC-CCGCGCGCCGGCAGACACAG-3') or NF1 (5'-GATCTTATTGT-GATTGAGGCCAATATGAG-3') were chemically synthesized (Biosearch 8700; Millipore, Bedford, MA).

**Transient Transfections and CAT Assays**

RCECs were grown either on plastic or on tissue culture plates coated with FN, LM, or CIV at various densities (from 5 × 10³–1 × 10⁴ cells/cm²) or at 3.5 × 10⁴ cells/cm², as specified in the figure legends, and transfected 24 hours later (48 hours when cells were grown on the ECM), by using a polycationic detergent (Lipofectamine; Invitrogen-Gibco, Burlington, ON, Canada), according to the manufacturer’s recommendations. When indicated, inhibitors of the intracellular signaling pathways were added 3 hours after transfection. For the antibody blocking experiment, RCECs were incubated on ice for 20 minutes with increasing concentrations (0–500 ng) of a ω5 integrin-specific, blocking monoclonal antibody (CD49e; BD Biosciences-PharMingen, Mississauga, ON, Canada). Cells were then incubated for 48 hours before transfection on tissue culture plates single-coated with BSA, FN (5 µg/cm²), or CIV (3 µg/cm²), or on plates double-coated with FN and CIV. Each transfected plate received 1 µg of the PCR-CAT test plasmids and 1 µg pXCGHS. Levels of CAT activity for all transfected cells were determined as described and normalized to both the amount of human growth hormone (hGH) secreted into the culture media (and assayed by using a kit for quantitative measurement of hGH (Immuno-corp, Montréal, QB, Canada) and the amount of nuclear proteins from the extract used. Each single value was expressed as 100 × (% CAT in 4 hours)/100 µg protein/ng hGH. The value presented for each plasmid transfected corresponds to the mean of at least three separate transfections done in triplicate.

**Nuclear Extracts and Electrophoretic Mobility Shift Assays**

Crude nuclear extracts were prepared as described from RCECs grown either on BSA- or FN-coated, 175 cm² culture flasks, and with or
without addition of inhibitors of the cell-signaling pathways and dialyzed against DNasel buffer (50 mM KCl, 4 mM MgCl₂, 20 mM K₃PO₄ [pH 7.4]), 1 mM β-mercaptoethanol, and 20% glycerol). The protein concentration from each of the nuclear extracts was determined by the Bradford procedure and precisely validated through densitometric analysis (BioImage, visage 110; Genomic Solutions, Ann Arbor, MI) for all the extracts used. The concentration of each extract was then precisely adjusted and a sample from each extract loaded once again on a second gel and further stained with Coomassie blue to ensure uniformity among the various extracts prepared. Extracts were then kept frozen in small aliquots at −80°C until use.

Electrophoretic mobility shift assays (EMSA) were performed by incubating 4 × 10⁶ cpm-labeled probe consisting of the Sp1 oligonucleotide 5’ end labeled with ³²P, with 5 µg nuclear proteins in the presence of 25 ng of poly(dI-dC) (Pharmacia-LKB; Thermo Electron Corp., Waltham, MA) in buffer D (5 mM HEPES, 10% glycerol, 0.05 mM EDTA and 0.125 mM phenylmethylsulfonyl fluoride [PMSF]). Incubation proceeded at room temperature for 5 minutes, and DNA-protein complexes further separated by gel electrophoresis through a 10% native polyacrylamide gel run against Tris-glycine buffer as described.¹¹ Gels were dried and autoradiographed at −80°C. Competition experiments in EMSA were conducted as above, except that a 500-fold molar excess of either the Sp1 or NF-I unlabeled oligonucleotide was added to the reaction mixture as a competitor. Supershift experiments in EMSA were also conducted as just described, except that 3 µl of a polyclonal antibody directed against either Sp1 or NF-I (both from Santa Cruz Biotechnology, Santa Cruz, CA) was added to the proteins before addition of the probe.

### SDS-PAGE and Western Blot

Either 20 µg nuclear extracts (PARP, Sp1, and Sp3 blots) or 85 µg total proteins (Erk1/2 and P38 blots) were added to 1 volume of sample buffer (6 M urea, 65 mM Tris [pH 6.8], 10% [vol/vol] glycerol, 1% SDS, 0.00125% [wt/vol] bromphenol blue, and 300 mM β-mercaptoethanol) and then size-fractionated on a 10% SDS-polyacrylamide minigel before being transferred onto a nitrocellulose filter, blotted as described and then exposed to (1) rabbit polyclonal antibodies (all Abs used at 1:5000 dilution) raised against Sp1, Sp3, NF-I (Santa Cruz Biotechnology, Inc.), total Erk1/2 or phospho Erk1/2 (Calbiochem-Cedarlane, Hornby, ON, Canada), total P38 (Cedarlane) or phospho-P38 (Cell Signaling Technology) or (2) a monoclonal antibody raised against bovine PARP²⁵(C-2-10 Ab bought from Guy Poirier, Unit of Health and Environment, CHUL Research Center, Quebec, Canada; 1:10 000 dilution). After incubation for 1 hour at RT in a 1:10 000 dilution of a peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory-Bio/Can Scientific, Mississauga, ON, Canada), immunoreactive complexes were revealed with a Western blot detection kit (GE Healthcare, Baie d’Urfé, QB, Canada), and autoradiographed. When indicated, densitometric analyses were performed. Data are presented as the mean ± SE. Student’s t-test was used to assess the influence of ECM components (FN, CIV, and LM) versus BSA on the activity directed by the rPARP-1 promoter.

### Statistical Analyses

Data are presented as the mean ± SE. Student’s t-test was used to assess the influence of ECM components (FN, CIV, and LM) versus BSA on the activity directed by the rPARP-1 promoter.
whose absolute amount of protein decreased on growing cells. However, CAT activity increased by approximately threefold when cells are plated at 2.5 \times 10^4 cells/cm² and reached its highest activation level (a near 10-fold increase) at 3.5 \times 10^4 cells/cm². The stimulatory influence of FN was totally lost as cell density was increased further to 4.5 \times 10^4 cells/cm². Of note, the positive influence of FN began turning into a negative regulatory influence at 6.5 \times 10^4 cells/cm² and reached an impressive 20-fold repression at 1 \times 10^5 cells/cm².

**The Sp1/NF-I Ratio in RCECs Grown on FN**

Transcription directed by the rPARP-1 promoter rely on both the positive regulatory influence of Sp1 and the negative regulatory action of the transcription factor NF-I, which competes with Sp1 binding as both transcription factors possess overlapping target sites in the rPARP-1 promoter.\(^1\rightarrow 5\) As shown on Figure 2A (left-side), incubation of nuclear extracts from subconfluent RCECs with a § end-labeled oligonucleotide bearing the high-affinity binding site for Sp1 yielded two DNA–protein complexes with formations found to be specific as the unlabeled Sp1 oligomer, but not a similar oligonucleotide bearing the target sequence for NF-I competed successfully with both. Supershift experiments in EMSA conducted through the further addition of antibodies directed against either Sp1 or Sp3 to the reaction mixture showed that the slow-migrating, more intense complex contains both Sp1 and Sp3 whereas the weaker, faster-migrating complex contained only Sp3. The addition of both the Sp1 and Sp3 antibodies (Sp1/Sp3 Ab; Fig. 2A, right-side). Its formation was found to be specific as its binding was successfully blocked by the NF-I but not by the unrelated Sp1 unlabeled oligomer. Furthermore, addition of a polyclonal antibody that recognizes any of the NF-I isoforms supershifted almost entirely the NF-I-DNA complex in EMSA. Therefore, RCECs express both Sp1/Sp3 and NF-I at subconfluence.

We then examined whether any quantitative alteration in the level of both Sp1 and NF-I may account for the opposite regulatory actions of FN on sub- (strong activation) and post-confluent (strong repression) RCECs. As shown in Figure 2B, FN considerably increased the DNA binding properties of both Sp1 and NF-I at subconfluence, with an Sp1/NF-I ratio (2.82 ± 0.5) that favored the positive action of Sp1, as evaluated through the phosphoimager quantification of the labeled DNA–protein complexes on the EMSA. As cells progressed toward quiescence at 5-days after confluence, DNA binding corresponding to both transcription factors dramatically diminished in the absence of FN. This result is consistent with our previous observations that Sp1 is degraded, most likely by the proteasome, when cells are maintained for such a period after confluence.\(^3\rightarrow 7\) Maintaining RCECs at 5-days after confluence in the presence of FN restored binding of both transcription factors. However, the FN influence on NF-I binding was beyond that observed for Sp1 at postconfluence, and therefore considerably altered the Sp1/NF-I ratio (which declined to 0.46 ± 0.5). Although culturing RCECs on FN at subconfluence indeed increased the absolute amount of Sp1 (184± 48%), which appears as three discrete protein bands in Western blot (Fig. 2C) corresponding to various posttranslationally modified forms of Sp1 (phosphorylated or glycosylated Sp1), yet this increase did not reach that observed in the EMSA (52.7% ± 69%) indicating that additional posttranslational modifications likely alter the affinity with which Sp1 binds to its target sites in DNA. This is even more dramatic for NF-I, whose absolute amount of protein decreased on growing cells on FN at subconfluence (Fig. 2C), whereas its DNA binding ability increased considerably (Fig. 2B).

**α5β1 Integrin Dependency of the PARP-1 Promoter FN Responsiveness**

To demonstrate that the FN responsiveness of the rPARP-1 promoter was dependent on the signal transduction cascade activated by the binding of FN to the α5β1 integrin, an antibody-directed receptor interference assay was conducted. RCECs were first incubated with increasing concentrations of a blocking anti-α5 Ab and then seeded in wells single-coated

![Figure 2](image_url)
with either BSA or FN (5 μg/cm²), or double-coated with both FN (5 μg/cm²) and CIV (3 μg/cm²). The addition of CIV was required as adhesion of RCECs to FN is almost exclusively dependent on α5β1 and that blocking this integrin with the CD49e Ab totally prevents their adhesion to FN (data not shown). As RCECs express the α2β1 and α5β1 integrin receptors for collagen,56-58 cell adhesion could be maintained on CIV, even in the presence of the α5 blocking Ab. As shown in Figure 3, a 10-fold increase in rPARP-1 promoter function was observed in RCECs cultured on FN. The further addition of CIV did not change the FN-mediated increase in rPARP-1 promoter activity. However, exposing the cells to 50 ng of the CD49e Ab was sufficient to reduce FN responsiveness by 71%, whereas 500 ng totally prevented its positive influence on the rPARP-1 promoter. Figure 3, a 10-fold increase in rPARP-1 promoter function was significantly different from those cultured on BSA (P < 0.005; Student’s t-test).

Influence of CIV and LM on rPARP-1 Promoter Activity

Basal corneal epithelial cells normally rest on a basement membrane essentially made out of CIV and LM that contains very little FN, which is produced only when the corneal surface is damaged. We therefore examined whether both CIV and LM may alter the activity directed by the rPARP-1 promoter when coated on the culture wells before seeding of RCECs. FN considerably improved proliferation of RCECs within the first 48 hours (cells grown on FN covered approximately 80% of the culture well, whereas RCECs grown on BSA covered approximately 60% when seeded at 3.5 × 10⁴ cells/cm²) whereas LM dramatically reduced it (Fig. 4A). Cells grown on FN spread very rapidly within the first few hours, whereas those grown on BSA required 24 hours to spread out completely. A significant proportion of the cells had not spread yet after 48 hours when plated on LM. These growth characteristics were maintained at 5 days after seeding. RCECs had a less differentiated phenotype with much smaller cells when grown on FN than on BSA. Although all cells attached and spread on LM 5 days after plating, they covered only 40% to 50% of the culture well. No growth alteration was observed with CIV, either 2 or 5 days after seeding (Fig. 4A).

We then transfected both PCR3 and PCR3/F2F3F4m into subconfluent RCECs seeded at 3.5 × 10⁴ cells/cm² on culture wells coated with BSA or with FN, LM, or CIV. Again, FN strongly increased PCR3 activity (–14-fold), whereas neither LM nor CIV had any influence on the basal rPARP-1 promoter activity when coated individually (Fig. 4B). Combining FN with CIV (FN+CIV) or with both CIV and LM (FN+CIV+LM), did not significantly alter the FN responsiveness of the rPARP-1 promoter. On the other hand, the positive influence FN exerted was entirely lost when similar experiments were conducted using the Sp1-mutated construct PCR3/F2F3F4m.

Cell-Signaling Pathways Activated by FN

Although the basal PARP-1 gene promoter shares many characteristics across different species (mouse, rat, and human), it remained to be established whether FN would similarly affect expression of the endogenous PARP-1 gene expressed by RCECs. Growing RCECs on FN (5 μg/cm²) considerably in-
increased the expression of endogenous PARP-1, as revealed by Western blot analysis (Fig. 5). Increased expression of PARP-1 on FN correlated with a dramatic increase in Sp1 expression and in a moderate increase in the expression of Sp3.

To decipher which of the MAPK, P38, and PI3K pathways account for the increase in PARP-1 expression resulting from the binding of FN to 

RCECs were then cultured at subconfluence on FN-coated culture plates either alone or in the presence of inhibitors (20 mM each) of the MAPK, PI3K, and P38 intracellular-signaling pathways. The MEK-kinase inhibitor PD98059 has been shown to be a very potent inhibitor of the MAPK pathway, whereas both wortmannin and SB203580 are currently used to inhibit the PI3K and P38 pathways, respectively. Total cell extracts were prepared and analyzed in Western blot experiments. As shown in Figure 6, expression of endogenous PARP-1 was considerably reduced in cells cultured on FN-coated plates in the presence of either PD98059 or wortmannin but not with SB203580. None of the three inhibitors had any influence on the level of both inactivated and phosphorylated P38.

We then conducted EMSA experiments on the total cell extracts just described, to confirm whether the changes in the expression of Sp1

FIGURE 5. Western blot analyses of proteins from RCECs grown on FN. Total cell extracts were prepared from subconfluent RCECs cultured on plates coated either with BSA or FN and examined in Western blot (75 μg for PARP-1, Sp1, P38, and P-P38; 20 μg for Erk1/2 and P-Erk1/2), with monoclonal antibodies directed against PARP-1, total ERK1/2 (ERK1/2), phosphorylated ERK1/2 (P-ERK1/2), total P38 (P38), and phosphorylated P38 (P-P38) or polyclonal antibodies against Sp1 and Sp3. The position of the 120- and 40 kDa proteins used as molecular mass markers is indicated.

FIGURE 6. Western blot and EMSA analyses of proteins from RCECs grown on FN, with or without inhibitors of the signaling pathways. (A) RCECs were grown on FN-coated plates, alone (FN) or in the presence of PD98059 (FN+PD), wortmannin (FN+W) or SB203580 (FN+SB). Total cell (for PARP-1, Sp1, and Sp3) or nuclear extracts (for ERK1/2, P-ERK1/2, P38 and P-P38) were then prepared and examined by Western blot with the antibodies from Figure 5. Data from one of four similar experiments are presented. (B) Crude nuclear proteins from RCECs grown on FN alone (lane 3) or in the presence of PD98059 (PD; lane 4), wortmannin (W; lane 5), or SB203580 (SB; lane 6) were incubated with an Sp1-labeled probe, and the formation of DNA-protein complexes was examined by EMSA. Nuclear proteins from RCECs grown on BSA were also used as the negative control (lane 2, BSA). The position of both the Sp1 and Sp3 complexes is shown, along with that of the free probe (U). P, labeled probe without nuclear proteins (lane 1).
Sp1 protein level also translate into corresponding alterations in its DNA binding properties. Consistent with the results shown in Figure 2A, growing RCECs on FN-coated plates increased the DNA binding ability of Sp1 toward its target site (Fig. 6B; compare lanes 2 and 3). However, FN responsiveness was totally lost when cells were grown with either PD98059 (lane 4) or wortmannin (lane 6), as the Sp1 labeled probe signal returned to that observed when RCECs are grown on BSA (lane 2). Again, SB203580 had no influence at all on the FN-induced level of Sp1 binding.

The functional significance of inhibiting the FN/α5β1-mediated signal transduction pathways on PARP-1 promoter activity was then assessed by transfecting PCR3 and PCR3/F2/F3/F4m into subconfluent RCECs plated either on BSA or FN-coated culture plates, and grown with PD98059, wortmannin, or SB203580. Again, PCR3 responded very nicely to the presence of FN on the culture plates (Fig. 7A) whereas FN responsiveness was totally abrogated when all three Sp1 sites were mutated in PCR3/F2/F3/F4m. As expected, both PD98059 and wortmannin severely and totally impaired FN responsiveness of the rPARP-1 promoter, respectively, but had no influence when PCR3 was replaced with PCR3/F2/F3/F4m. In an unexpected result, SB203580 totally blocked the FN responsiveness of the PCR3 construct. However, it also dramatically reduced the basal level directed by PCR3/F2/F3/F4m (14-fold reduction), even though all three Sp1 sites were mutated. To investigate further this Sp1-independent inhibition of the P38 signaling pathway by SB203580, both PCR3 and PCR3/F2/F3/F4m were cotransfected into RCECs grown on BSA- or FN-coated plates, along with a recombinant construct (pCMV-Flag-P38[AGF]) that encode high levels of expression of a dominant negative form of P38 (P38[AGF]). As with SB203580, overexpression of P38[AGF] entirely suppressed FN responsiveness directed by wild-type PCR3 and also considerably repressed (10-fold) the activity directed by the Sp1-mutated derivative PCR3/F2/F3/F4m (Fig. 7B).

**DISCUSSION**

When the corneal epithelium is injured, local changes in the basement membrane, mostly characterized by the massive, transitory secretion of FN, occur within the first few hours after the damage. This newly synthesized FN-enriched basement membrane has been postulated to serve as a temporary matrix for the attachment and migration of the basal epithelial cells that border the injured area, a process dictated in part by the interaction of FN with its corresponding membrane-bound integrin receptors. Engagement of integrins with their corresponding ligands then triggers the activation of a variety of signal-transduction pathways that alter the pattern of genes expressed by the epithelial cells bordering the damaged area of the cornea. In this study, we provided evidence that PARP-1 belongs to those genes with expression that is altered by FN in highly proliferative RCECs. The FN responsiveness of PARP-1 gene expression was shown to rely on dramatic changes in the affinity with which the transcription factor Sp1 binds to its target sites in the PARP-1 basal promoter. This Sp1-dependent increase in PARP-1 promoter activity was shown to result from the activation of either the MAPK or the P38K pathway in response to the engagement of the α5β1 integrin receptor by FN.

FN responsiveness of the rPARP-1 promoter was found to be cell-density-dependent, peaking at $3.5 \times 10^4$ cells/cm$^2$, a seeding density that corresponded to ~80% coverage of the culture plate at the moment the cells were transfected. Such a cell coverage of the culture surface also correlated perfectly with the density at which both endogenous PARP-1 and Sp1 reached their best possible level of expression (as revealed by Western blot analyses) and DNA binding properties (as revealed by EMSAs for Sp1). Expression of Sp1 was shown to disappear very rapidly, often to nondetectable levels in certain types of cells, as primary cultured cells reached growth arrest at postconfluence in the absence of FN. However, maintaining RCECs at postconfluence in the presence of FN restored, although to a lower level, the expression of Sp1 in these cells. Yet, rPARP-1 promoter activity failed to be properly activated as its transcription became negatively regulated by FN at a high cell density. This reversion of the positive influence of FN into a negative influence when RCECs are plated at a high density ($1 \times 10^6$ cells per 35-mm well) could have resulted from the posttranslational modification, probably...
phosphorylation, of transcription factors other than Sp1 that are also necessary to maintain proper rPARP-1 promoter function. One such candidate transcription factor might as well belong to the NF-I family.65 Indeed, the activity directed by the rPARP-1 promoter has been recently shown to be negatively regulated by NF-I.54,55 Although NF-I appears to be transcriptionally inert by itself as it possesses no intrinsic activity in the regulation of this gene system, its negative influence results from the fact that it competes with Sp1 for the availability of a promoter composite element that bears overlapping target sites for both these transcription factors.55 This particular type of overlapping arrangement for both the Sp1 and NF-I target sites in which NF-I negatively influences gene expression by preventing Sp1 from interacting with its binding site is not unique to the rPARP-1 promoter as it has also been reported for both the collagen alpha1(I)46 and the platelet-derived growth factor (PDGF)-A genes.67 An interesting observation was that, whereas the DNA binding of Sp1 (and to a lower extend, its expression too) decreased in postconfluent cells grown in the presence of FN, that of NF-I increased considerably. Consequently, the Sp1/NF-I ratio of activities, which clearly favored the positive action of Sp1 at subconfluence (1.82 ± 0.5), switched toward NF-I interference at postconfluence (0.46 ± 0.5). Yet, the possibility remains that transcription factors other than NF-I might account for the repression of the rPARP-1 promoter function. Although such a negative action involved in the regulation (possibly negatively) of basal PARP-1 expression, whose transcription has been reported to be decreased in FN-mediated growth arrest of both normal and cancer cells. Studies by Arita et al.68 and Clarke et al.69 have provided evidence that Sp1 suppresses cell growth by decreasing the expression of the cell cycle inhibitor p21/WAF-1. This LM-mediated growth arrest appears to rely on the cytoplasmic domain of the β1 integrin subunit from the LM-binding integrin α6β4, which has been shown to be linked to a signaling pathway that induces expression of p21. Besides p21, laminin-5 has also been reported to be coexpressed with the tumor suppressor p16 in epidermal keratinocytes at the migrating front of healing wounds, thereby causing growth arrest of migratory keratinocytes that lead to wound epithelialization.70

Binding of ECM components with their corresponding integrin receptors triggers the activation of intracellular signaling mediators, such as focal adhesion kinase (FAK); the MAPKs Erk1/2, JNKs, and p38; and Rho family GTPases, such as RhoA, Rac1, and CDC42 (for reviews, see Refs. 71, 72). Activated FAK will, in turn, activate Erk1/2 kinases through the Ras/Raf-1/NF-I-MAPK (Erk) pathway. However, Erk1/2 can also become activated through the activation of PKC/Raf1 by PI3K.60,73 Activation of Erk1 and Erk2 through phosphorylation causes their translocation to the nucleus, where they have been reported to phosphorylate and activate several transcription factors, such as LSF, ETS1, ELK, c-Jun, c-Myc, and PEA3,74-79 as well as Sp1.80-82 Our pharmacological inhibition studies indicate that binding of FN to its integrin receptor α5β1 increases PARP-1 gene expression in migrating and highly proliferative subconfluent RCECs, a model that compares favorably to corneal wound healing, by activating Erk1/2 through the MAPK or the PI3K, but not the P38, pathway. This finding is consistent with the increased DNA-binding properties of Sp1, a recognized downstream target of Erk1/2, observed when RCECs were grown on FN, and with the corresponding increase in PARP-1 expression, whose transcription has been reported to be primarily dependent on the positive influence of both Sp1 and Sp3 in vitro.80-81 Of interest, and in agreement with our results, scratch-wound–induced migration of human endothelial cells in a shear stress model, a process shown to depend strictly on the interaction of the α5β1 integrin with FN, was shown to rely on activation of both the MAPK ERK 1/2 and PI3K.83 PI3K has been reported as necessary for integrin–stimulated activation of the MAPK cascade and the serine/threonine kinase Akt.66 The PI3K/Akt pathway triggers a cascade of responses involved in cell survival, proliferation, and growth (reviewed in Refs. 84,85). Although both ERK1 and ERK2 are well recognized as the major downstream effectors of the MAPK pathway, those from Akt are just beginning to be identified. They include the proapoptotic protein BAD,86 glycogen synthetase kinase-3β (GSK-3β,87 the newly identified target protein NAG-1 (nonsteroidal anti-inflammatory drug-activated gene88), as well as transcription factors such as NFκB, and Forkhead (reviewed in Ref. 85). At the present time, we have no evidence as to whether Akt becomes activated as a consequence of the α5β1 integrin occupancy by FN in primary cultured RCECs.

Rather surprisingly, bypassing P38 signalization through either the overexpression of a dominant negative form of P38 or the pharmacological inhibition of P38 abolished FN responsiveness of the rPARP-1 promoter irrespective of whether the Sp1 sites were mutated (Fig. 7). These results suggest that FN may have triggered the activation of P38 (although we did not see it in the experiments shown in Figs. 5 and 6A) and thereby altered a transcription factor other than Sp1 that is normally involved in the regulation (possibly negatively) of basal rPARP-1 promoter function. Although such a negative action would be well suited for NF-I, its phosphorylation has been shown to improve its DNA binding rather than to decrease it. NF-I has been reported to interact directly with a component from the p300/CBP coactivator complex—an interaction abrogating the NF-I/C-mediated repression of the MMTV promoter.89 Recently, p300/CBP was identified as a target of activated P38, with its phosphorylation preceding its degradation by the proteasome.90 It is then conceivable that the NF-I proteins sequestered into the p300/CBP complexes would also be subjected to degradation by the proteasome on activation of P38, being thus unavailable to regulate the expression of their target genes. Binding of NF-I to the PARP-1 promoter would then be required on activation of P38 and would translate into an increase in PARP-1 expression. This would be consistent with a functional requirement for PARP-1 in pathologic stresses that are induced by radical oxygen species, hypoxia, and proinflammatory cytokines as they mediate their influence through activation of the P38 signaling pathway.90 Hypoxia and oxygen species are known inducers of PARP-1 activity, as they both damage DNA (reviewed in Ref. 91).

Much evidence points toward a major function for PARP-1 in tissue damage.91 Tissue insults lead to DNA damage, which can arise from the formation of nitric-oxide derivatives such as...
peroxynitrite.\textsuperscript{92} As a consequence, PARP-1 becomes over-activated and may lead to an important depletion in its substrate NAD\textsuperscript{+}. In response to the NAD\textsuperscript{+} depletion, the cell's attempt to resynthesize this substrate leads to a depletion of ATP and triggers the cell to die from energy loss. This process allows for the elimination of cells that are too damaged to progress through the many steps of the wounded process. Indeed, anterior stromal keratocytes undergo apoptosis in response to corneal epithelial injury in a proportion that may range from 0.9\% to 5.1\%, depending on the surgical procedure selected.\textsuperscript{95} However, unlike stromal keratocytes, corneal epithelial cells have been reported to be resistant to apoptosis, as only a small proportion of the cells lost from the surface of the cornea through shedding enter apoptosis.\textsuperscript{94} Growth factors, such as hepatocyte growth factor (HGF), have been shown to confer cytoprotection on corneal epithelial cells by preventing them from progressing into apoptosis through the activation of the PI3K/Akt-1/Bad- but not the ERK1/2-mediated signal transduction pathways.\textsuperscript{95} Of note, studies by Hoyt et al.\textsuperscript{96-97} provided evidence that engagement of \(\beta1\)-integrins can prevent acute DNA breakage caused by a variety of unrelated agents, such as the antitumor agent bleomycin, by dramatically reducing poly(ADP-ribose) synthesis by PARP-1 in response to DNA damage. Integrin clustering has been proposed to alter the chromatin structure by a PARP-modulated nuclear response.\textsuperscript{98} As FN increased PARP-1 expression in RCECs without any apparent alteration in its activation status (data not shown), it is expected that no depletion in NAD\textsuperscript{+} or ATP occurred under such culture condition. Then, what physiological advantage would such an increase in PARP-1 protein confer to RCECs during wound healing? One possible way by which PARP-1 may contribute to wound healing without the need for the cell to progress toward apoptosis is through alteration of transcription factors that regulate genes whose encoded products are necessary for cell adhesion and migration. Gene disruption or pharmacological inactivation of PARP-1 has been reported to reduce the cytokine-mediated expression of ICAM-1, P-selectin, and E-selectin, as well as mucosal addressin cell adhesion molecule (MAdCAM)-1 in human umbilical vein endothelial cells.\textsuperscript{99} PARP-1 has been reported to modulate the expression of the integrin CD11a in the migration of microglial cells after brain injury.\textsuperscript{100} PARP-1 may do so either by directly interacting with transcription factors, as shown for YY-1, AP-2, B-MYB, Oct-1, TEF-1, and NF-\(\kappa\)B, or through their poly(ADP-ribose)lation, as evidenced for p53, fos, NF-\(\kappa\)B, and both RNA polymerases I and II (reviewed in Ref. 101). Although PARP-1 has been most often reported to interface with the positive regulatory influences mediated by these transcription factors, some evidence suggests that it may also act as a coactivator or enhancer factor and thereby promote gene transcription.\textsuperscript{102,103} Target sites for some of these transcription factors (AP-1, AP-2, B-MYB, and NF-\(\kappa\)B) were identified in many integrins' promoters. Both AP-1 and -2 are of particular interest, as binding sites for these transcription factors have been identified in the promoter of the \(\alpha1\), \(\alpha5\), and \(\alpha6\) integrin gene subunits\textsuperscript{26,104,105}; and the expression of these transcription factors has been reported to be increased during corneal wound healing.\textsuperscript{37,106,107} The transcription factor Pax-6, necessary for proper development of many eye structures including the cornea, the lens, and the retina, is also worth mentioning, as its expression has been demonstrated to be under the influence of PARP-1.\textsuperscript{108} Pax-6 expression is increased at the migrating edge as the epithelium resurfaces the cornea after injury,\textsuperscript{109} and may contribute to corneal wound healing by modulating the expression of Pax-6 responsive genes, which comprise those encoding the integrin subunits \(\beta1\), \(\alpha4\), and \(\alpha5\).\textsuperscript{106,110,111} It is interesting that activation of the Sp1 DNA-binding activity by TNF-\(\alpha\) or LPS requires PARP-1 activity, as S1 activation has been found to be lower in PARP-1 \textsuperscript{\textendash} the corneal epithelium is obviously a process with effectiveness that is dependent on the intracellular signals transduced by the binding of membrane-bound integrins to components from the ECM such as FN. PARP-1 may turn out to be a major component of the wound-healing response by being overexpressed during the proliferative burst that characterizes this process, although the precise mechanism through which this is accomplished remains elusive.

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