Inhibition of the Phosphatidylinositol-3-Kinase Pathway Abrogates Polyinosinic:Polycytidylic Acid–Stimulated Hyaluronan-Mediated Human Mucosal Smooth Muscle Cell Binding of U937 Monocytic Cells

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The origin of inflammatory bowel disease (IBD) is unknown and likely to be multifactorial. Our laboratory has established that in human mucosal smooth muscle cells (M-SMCs), cellular stress induced by virus or the viral mimic double-stranded RNA (polyinosinic:polycytidylic acid [poly I:C]) increases cell surface hyaluronan (HA) deposition and the formation of long cable-like structures of HA that are important for leukocyte attachment. Since leukocyte accumulation and hyperplasia of the M-SMCs are characteristic pathological changes observed in IBD patients, and phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways play established roles in cell survival, we investigated whether this pathway is involved in this unique HA-mediated leukocyte attachment. Poly I:C–stimulated M-SMCs bind significantly more monocytic cells than untreated cells and this response was inhibited in a dose-dependent manner by treatment with the PI3K inhibitor, LY294002. Since Akt is a critical downstream regulator of PI3K, we investigated the phosphorylation status of Akt in M-SMCs after treatment with poly I:C for 1 h and found that Akt was phosphorylated, but the phosphorylated Akt band was undetectable in LY294002 plus poly I:C–treated cultures. Confocal microscopy of M-SMCs stained for HA revealed that HA cable formation after poly I:C treatment was abrogated by LY294002. These results demonstrate that poly I:C–stimulated M-SMCs phosphorylate Akt, produce HA cables, and promote HA-mediated leukocyte adhesion through a PI3K/Akt-dependent manner.

Introduction

THE PATHOGENESIS OF CHRONIC inflammatory conditions such as inflammatory bowel disease (IBD), asthma, and atherosclerosis is not well understood. IBD initiation could be due to exposure of environmental factors that alter the normal homeostasis within the gut in genetically susceptible people. A recent concept of IBD suggests that it is a result of a complicated interplay of multiple factors that include nonimmune and immune cell interactions. Inflammation may originate by various stress-inducing agents that affect cell proliferation and differentiation, as well as alterations in cellular gene expression, by modulating several signaling pathways.

Hyaluronan (HA) is a glycosaminoglycan composed of glucuronic acid and *N*-acetyl-*D*-glucosamine, and is a major component of extracellular matrix (ECM). Our laboratory has demonstrated that HA is upregulated in inflamed colon from IBD patients and is correlated with *in vitro* cellular stress induced by virus or the viral mimic, synthetic double-stranded RNA (polyinosinic:polycytidylic acid [poly I:C]), in

cultured mucosal smooth muscle cells (M-SMCs) (de la Motte and others 1999, 2003). We established that *in vitro*, tunicamycin-induced endoplasmic reticulum (ER) stress or dextran sulfate sodium (DSS) treatment increases HA deposition in a unique way to form long cable-like structures that are important for leukocyte attachment (Majors and others 2003). We showed recently that signal transducer and activator of transcription-1 (STAT1) plays an important role in poly I:C–stimulated HA deposition in mouse colonic SMCs and is important for leukocytes attachment (Bandyopadhyay and others 2008). We also demonstrated that DSS-induced colon damage in STAT1 null mice is less severe than the damage in the colon of wild-type mice (Bandyopadhyay and others 2008). The phosphorylation of 701-tyrosine and 727-serine STAT1 residues is necessary for maximum transcriptional activation of STAT1-dependent genes (Darnell and others 1994; Stark and others 1998; Decker and Kovarik 2000), and we demonstrated that poly I:C treatment in M-SMCs phosphorylates STAT1 at both tyrosine and serine residues (Bandyopadhyay and others 2008). STAT1 is shown to be 727-serine-phosphorylated at the C-terminal end by

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phosphatidylinositol-3-kinase (PI3K) and also by mitogen-activated protein kinase (MAPK) (Kovarik and others 1999; Nguyen and others 2001). Many agents, including growth factors, cytokines, and virus infections, activate PI3K (Datta and others 1999; Brazil and others 2002; Cantley 2002; Fujita and others 2002; Downward 2004; Franke and others 1997, 2003). Serine-threonine protein kinase Akt, also known as protein kinase B, plays roles in normal and pathophysiological conditions and acts as a major downstream signaling molecule in the PI3K pathway (Brazil and others 2002; Cantley 2002). The PI3K/Akt pathway plays a key role in the suppression of apoptotic cell death induced by various stimuli of apoptosis including various stresses (Franke and others 1997, 2003; Datta and others 1999). Akt is considered a multifunctional protein kinase rather than only a regulator of cell survival because downstream effectors of Akt are involved in varied aspects of cellular functions (Downward 2004; Hu and others 2004; Puri and others 2004; Tessner and others 2004; Amaravadi and Thompson 2005).

Since accumulation of mononuclear leukocytes and hyperplasia are characteristic pathological changes observed in the intestinal mucosa of IBD patients, we investigated whether the PI3K/Akt pathway is involved in this unique poly I:C-stimulated HA-mediated leukocyte attachment in primary human M-SMCs. In this study, our experimental results demonstrate that blocking the PI3K/Akt pathway drastically inhibits poly I:C-induced HA-mediated cell adhesion.

Materials and Methods

Materials

Cell culture media and additives were obtained from our institute core facility. Fetal bovine serum was from Bio-Witaker; poly I:C and dimethyl sulfoxide (DMSO) were from Sigma; Akt, STAT1, PI3K, and phosphorylated Akt antibodies were from Cell Signaling Technology; protease inhibitors and LY294002 were from Calbiochem.

Cell culture

The techniques of cell isolation, culture, and adhesion assays of human M-SMCs were described in our earlier publications (de la Motte and others 1999, 2003; Majors and others 2003). Briefly, after enzymatic digestion and isolation, M-SMCs were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 100 mg/mL of streptomycin, and 0.25 mg/mL of Fungizone in a humidified cell culture incubator at 37°C with 5% CO₂ unless otherwise indicated. Approximately 70%–80% confluent cell cultures were used and all experiments were done within the third passage of cells in culture. Cells were treated with LY294002 dissolved in DMSO at indicated concentrations for 1 h prior and during the treatment, either without or with 50 µg/mL poly I:C for 22 h unless otherwise mentioned. Control cells were treated with the medium containing the same volume of DMSO as LY294002.

Leukocyte binding assay

Binding of U937 and THP1 (human monocytic cell line) cells to M-SMCs was measured either without or with ⁵¹Cr-labeled U937 cells according to our previously published

procedure (de la Motte and others 1999; Majors and others 2003). The medium was removed and cells were washed once with the medium before the addition of monocytic cells for the binding assay. One of the representative results from 3 independent experiments is shown. Cell binding values were presented as the mean data from at least triplicate wells plus/minus the standard error.

Fluorescence histochemistry for confocal microscopy

Sterile cover slips were placed within 6-well plates and cells were seeded onto cover slips. Cells were treated with DMSO as a vehicle control for LY294002 or 50 µg/mL poly I:C with or without LY294002 as indicated in figures. After 22 h of treatment, cover slips were fixed in cold methanol at –20°C for 10 min, and then the methanol was removed completely and air-dried at room temperature. Fluorescent staining for HA was performed using biotinylated HA binding protein as described previously (de la Motte and others 2003). The HA binding protein was omitted in assays serving as negative control. PI3K and STAT1 antibodies were used according to manufacturer's suggestion. We used secondary fluorescein-isothiocyanate-conjugated Streptavidin for HA detection and also 4,6-diamidino-2-phenylindole for nuclei staining. The slides were examined by Leica TCS-SP laser-scanning confocal microscopy (Bandyopadhyay and others 2008).

Immunoblot

Western blots were done with equal amounts of protein per lane, as measured by the BioRad protein assay reagent, according to standard procedure. Protein extracts were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride transfer membrane at 4°C by a standard procedure (Bandyopadhyay and others 2000, 2008). Antibodies to both phosphorylated Akt and Akt were used according to the manufacturer's protocols. Immunodetected proteins were observed using the ECL kit from Amersham following manufacturer's protocol.

Results

Inhibition of U937 cell attachment to poly I:C-stimulated human M-SMCs by PI3K inhibitor

We previously showed that cellular stress induced by virus or poly I:C, or by chemicals such as DSS or tunicamycin stimulates HA deposition in primary SMCs in a unique way that facilitates leukocyte adhesion in a HA-dependent manner (de la Motte and others 1999, 2003; Majors and others 2003). In this first experiment, we tested whether PI3K inhibition, by a known chemical inhibitor, LY294002, had an effect on HA-mediated U937 or THP1 cell adhesion to M-SMCs. Human M-SMCs were plated in 24-well plates for qualitative (panels A through E) and quantitative (Fig. 1a panel F) binding assays using unlabeled and radiolabeled U937 cells, respectively. Binding of U937 and THP1 cells to human M-SMCs is shown in Fig. 1a and b, respectively. Panel B in Fig. 1a and b shows that substantially higher numbers of monocytic cells are bound to poly I:C-treated M-SMCs compared to only DMSO-treated M-SMCs (panel

FIG. 1. Inhibition of U937 cell attachment to poly I:C-stimulated human M-SMCs by LY294002. (a): Cells were plated into 24-well plates and treated the next day for 22 h with (A) DMSO, (B) 50 μ g/mL poly I:C, (C) 50 μ M LY294002 dissolved in DMSO 1 h before and together with 50 μ g/mL poly I:C, or (D) 50 μ M LY294002 only. Cells were washed once with the medium and then unlabeled U937 cells were added into each well for attachment assay as described in the Materials and Methods section. (E) To determine the HA-mediated cell attachment, removal of HA-bound U937 cells in poly I:C-treated wells was achieved by treatment with hyaluronidase for 5 min. (F) Replicate parallel cultures in triplicates were treated similarly as (A) through (E), and quantitative binding assays were performed using 51 Cr-labeled U937 cells and the fold increase was presented as a graph with DMSO-treated cultures represented as one. (b): Panels (A) through (E) are same as described in (a) except that unlabeled THP1 cells were used instead of U937 cells. In panel (F), M-SMCs were treated with poly I:C for 22 h, and then cells were washed once, and unlabeled THP1 cells were added and incubated for 1 h at 4°C. Unbound cells were washed, and then cultures were fixed with methanol and processed for immunofluorescent detection of HA (green) and nuclei (blue). A 40 $\times$ magnification is shown with arrows indicating cable-like structures of HA. DMSO, dimethyl sulfoxide; HA, hyaluronan; M-SMCs, mucosal smooth muscle cells; poly I:C, polyinosinic:polycytidylic acid; THP1, human monocytic cell line.

FIG. 2. Dose-dependent inhibition of U937 cell attachment to poly I:C-stimulated human mucosal smooth muscle cells by LY294002. Cells were plated into 24-well plates, and the next day cells were treated with DMSO only or with LY294002 at 10, 20, 30, 40, and 50 μ M concentrations in the presence or absence of 50 μ g/mL poly I:C for 22 h as indicated. All *P* values were calculated by Student's *t*-test. $\otimes P$ 0.0068 versus untreated (DMSO); $*P > 0.08$ versus poly I:C treated; $**P < 0.009$ versus poly I:C treated; $^{\#}P > 0.07$ versus untreated (DMSO) also. The cell adhesion assay was performed using ^{51}Cr -labeled U937 cells as described in the Materials and Methods section. DMSO, dimethyl sulfoxide; poly I:C, polyinosinic:polycytidylic acid.

A). Panel C shows inhibition of poly I:C-induced cell attachment by 50 μ M LY294002 treatment compared to poly I:C only treated cells. LY294002 treatment (panel D) resulted in a similar number of attached U937 cells as compared to vehicle (DMSO)-treated cells (panel A). Similar binding of monocytic cells to M-SMCs was observed for cultures treated with DMSO or incubated with the medium alone. Removal of bound cells by hyaluronidase treatment verifies that monocytic cell attachment is mostly mediated by HA (panel E). In Fig. 1a panel F, quantitative assays in parallel cultures using ^{51}Cr -labeled U937 cells demonstrated that adhesion is increased >9-fold in poly I:C-treated cells (panel B) compared to vehicle-treated cells (panel A). LY294002 together with poly I:C treatment (panel C) severely reduced U937 cell binding and only about 2.4-fold induction is observed, which is similar to hyaluronidase-treated cells (panel E). As reported previously, this quantitative binding of U937 cell adhesion to M-SMCs shows that adhesion is mostly HA mediated (de la Motte and others 1999, 2003; Majors and others 2003). In Fig. 1b panel F, HA staining by fluorescence confocal microscopy shows that poly I:C-treated M-SMCs produce long HA-cable-like structures (green staining with arrow) that bind THP1 cells. It is worth mentioning that tumor necrosis factor- α (TNF- α)-induced adhesion of U937 cells to M-SMCs (via vascular cell adhesion molecule [VCAM]) was neither mediated by HA nor inhibited by the PI3K inhibitor, LY294002 (data not shown). These data indicate that the LY294002 compound prevents poly I:C-induced HA-mediated leukocyte adhesion but not VCAM-mediated adhesion. However, TNF- α -induced leukocyte adhesion, but not poly I:C-induced HA-mediated adhesion, was inhibited by a MAPK inhibitor (data not shown). These results indicate that inhibition of PI3K abrogates poly I:C-induced HA-mediated leukocyte attachment to human M-SMCs.

LY294002 treatment on poly I:C-stimulated M-SMCs dose dependently inhibits attachment of U937 cells

Next we investigated whether this inhibition of PI3K reduced the U937 cell attachment to poly I:C-treated M-SMCs in a dose-dependent manner. Cell adhesion assays were performed after cells were treated with a medium containing DMSO alone, or containing LY294002 dissolved in DMSO at concentrations of 10, 20, 30, 40, and 50 μ M, in the presence or absence of poly I:C. Results in Fig. 2 indicate that LY294002 treatment dose dependently inhibited poly I:C-stimulated U937 cell binding to M-SMCs. Maximum inhibition was observed at 50 μ M, although the inhibition was $\sim 75\%$ starting at 30 μ M. LY294002 treatment was not toxic to the cells, even at 50 μ M, and the binding of U937 cells to M-SMC in the presence of different concentrations of LY294002 only was similar to the binding observed with vehicle-treated cells. These results indicate that poly I:C-stimulated HA-mediated cell adhesion is reduced significantly by treatment with LY294002.

Poly I:C-induced HA accumulation and Akt phosphorylation are inhibited by LY294002

Since we showed previously that poly I:C induced the deposition of a unique HA structure that facilitates the binding of leukocytes, we investigated the effect of LY294002 treatment on poly I:C-stimulated HA deposition in human M-SMCs by fluorescent staining (de la Motte and others 1999, 2003; Majors and others 2003). Human M-SMCs were vehicle treated or treated with LY294002 either with or without poly I:C for 22 h, and HA staining was observed by confocal microscopy. Figure 3a shows that poly I:C treatment results in long cable-like structures of HA (green), and both LY294002 alone and together with poly I:C treatment diminished HA staining, and completely abrogated the long cable-like structures. This observation suggests that blocking PI3K inhibits the poly I:C-stimulated long cable-like HA structure formation in human M-SMCs. HA staining was less in LY294002-treated cells than in the vehicle-treated cells. Although PI3K/Akt pathways, in general, mediate signals for proliferation, in our *in vitro* experiments we did not observe a significant increase in the proliferation rate in isolated primary human colonic M-SMCs after poly I:C treatment (data not shown). Since Akt is downstream of PI3K, we decided to examine whether poly I:C treatment causes phosphorylation of Akt in human M-SMCs. Cell extracts were prepared from M-SMCs treated with poly I:C for 0, 15, 30, 60, or 120 min, to measure the Akt Ser-473 phosphorylation by Western blot using a specific antibody. Results demonstrate that Akt is phosphorylated as early as 15 min, maximum phosphorylation was observed at 1 h, and complete dephosphorylation occurs by 2 h after poly I:C treatment (Fig. 3b). Since LY294002 compound is a known PI3K inhibitor and blocks downstream Akt phosphorylation, we verified whether LY294002 treatment is effectively blocking Akt phosphorylation under our experimental conditions, and the results indeed demonstrated the inhibition of Akt phosphorylation (Fig. 3c). These results indicate that poly I:C stimulates Akt phosphorylation, and that the deposition of long HA structures by M-SMCs is inhibited by LY294002. Next we determined the effects of LY294002 on poly I:C-

inducible 727-serine phosphorylation of STAT1 in M-SMCs. To determine the 727-serine phosphorylation of STAT1, cells were treated for 2 h with or without poly I:C in the presence or absence of LY294002. Figure 3d shows minimal STAT1-serine phosphorylation in the untreated control cells, while substantial serine phosphorylation of STAT1 was observed in poly I:C-treated cells. This result indicates that LY294002 compound prevents the 727-serine phosphorylation of STAT1 induced by poly I:C in M-SMCs. We further investigated the staining pattern of PI3K and STAT1 after poly I:C

treatment using immunofluorescence confocal microscopy. The next day, after plating M-SMCs on cover slips within 6-well plates, cells were either treated with medium alone or with poly I:C for 0, 0.5, 1, or 2 h and then stained for PI3K (green) and STAT1 (red) as shown in Fig. 3e. The PI3K staining is more intense in poly I:C-treated cells than in untreated cells. Maximum staining of PI3K is observed 1 h after poly I:C treatment and diminished after 2 h of poly I:C treatment. The STAT1 staining results are similar to that for PI3K staining and is also maximum at 1 h poly I:C-treated

FIG. 3. Poly I:C-induced HA accumulation and Akt phosphorylation are inhibited by LY294002. Cells were plated onto cover slips and the next day treated with DMSO (designated as untreated in the figure) or with and without 50 μ M LY294002 in the presence or absence of 50 μ g/mL of poly I:C for 22 h (a). Cells were pretreated with LY294002 1 h before the start of poly I:C treatment. Cells were methanol fixed and stained for HA (green) and nuclei (blue) as described in the Materials and Methods section. Arrows indicate cable-like structures of HA. (b) Mucosal smooth muscle cells were treated with poly I:C in the presence of the 1%-serum-containing medium for the indicated times, and Western blotting was performed with 80 μ g protein in each lane using a specific antibody to phosphorylated Akt. The same blot was also used to determine total Akt using another specific antibody. (c) Cells were vehicle (DMSO) treated as a control for LY294002, or treated with 50 μ g/mL poly I:C in the presence or absence of 50 μ M LY294002 as indicated. Cells were pretreated with LY294002 1 h before the treatment of poly I:C. At the end of treatments, cellular extracts were prepared and Western immunoblots were performed to detect both phosphorylated Akt and (d) 727-serine STAT1 as indicated in the figures using ECL reagent. (e) Cells were treated as described in the figure and then methanol fixed. Cells were then stained for PI3K (green), total STAT1 (red), and nuclei with DAPI (blue). A 20 $\times$ magnification is shown. DAPI, 4,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; HA, hyaluronan; PI3K, phosphatidylinositol-3-kinase; poly I:C, polyinosinic:polycytidylic acid; STAT1, signal transducer and activator of transcription-1.

FIG. 3. (CONTINUED).

cells. The overlay pictures show that the PI3K and STAT1 colocalized maximally after 1 h of poly I:C treatment. This observation indicates an early association of PI3K and STAT1 in M-SMCs treated with poly I:C.

Discussion

We have previously reported that virus-treated M-SMCs bind mononuclear leukocytes via a HA-based mechanism (de la Motte and others 1999). The viral-mimic, poly I:C, is a synthetic double-stranded RNA that also induces HA-mediated leukocyte adhesion to M-SMCs (de la Motte and others 1999, 2003). HA-specific fluorescent staining and confocal microscopy revealed that the leukocyte-adhesive HA produced by stimulated M-SMCs is distinctly different from the cell coat HA produced by unstimulated cells (de la Motte and others 1999, 2003).

Inflammation and leukocyte attachment may occur through numerous pathways that are mainly triggered by cytokines and involve several proteins, including, but not limited to, nuclear factor-kappaB (NF- κ B), SMAD name is derived from combination of *c. elegans* SMA protein and *Drosophila* MAD proteins, members of the Janus kinase/

STAT protein family, and also the ER stress response protein, XBP1 (Darnell and others 1994; Stark and others 1998; Puri and others 2004; Feinberg and Jain 2005; Lin and Karin 2007; Kaser and others 2008). Our study and those of others indicate that ER stress also contributes to the pathogenesis of chronic inflammatory conditions such as IBD (Majors and others 2003; Kaser and others 2008; Kaser and Blumberg 2009). Specifically, when ER stress was induced in M-SMCs with the prototypical ER stress agent, tunicamycin, we demonstrated that HA polymers are also deposited and the ECM becomes adhesive for leukocytes in a HA-dependent manner (Majors and others 2003). Our earlier work revealed that several cytokines used individually or in combination are incapable of stimulating leukocyte-adhesive HA production by M-SMCs (Bandyopadhyay and others 2008); however, some stress mechanisms can transduce signals through alternative pathways to activate target genes in the absence of ligand-receptor interactions (Dudley and others 2004). Emerging evidence suggests that STATs also mediate cellular responses to cell stress (Dudley and others 2004). While the STAT signaling pathway and activation is typically considered to require ligand-receptor binding, cellular stress has also been shown to likely cause activation of unconventional

STAT pathways, such as those that associate STAT with ancillary second messengers like PI3K (Dudley and others 2004). These findings suggest that some STATs can be phosphorylated near the carboxy terminus through the PI3K/Akt pathway or by members of the stress-induced MAPK family (Kovarik and others 1999; Nguyen and others 2001; Stephanou and others 2001). We have shown recently that poly I:C-stimulated colonic SMCs isolated from STAT1 null mice have drastically reduced HA-dependent U937 cell adhesion compared to wild-type cells, and that STAT1 was phosphorylated by poly I:C treatment at the critical Ser residue (Bandyopadhyay and others 2008). The PI3K/Akt pathway is recognized to play a key role in the suppression of apoptotic cell death induced by various stimuli of apoptosis, including ER stress, oxidative stress, and viral infection (Datta and others 1999; Brazil and others 2002; Cantley 2002; Fujita and others 2002; Shiojima and Walsh 2002; Downward 2004; Vantler and others 2005; Franke and others 1997, 2003). However, other downstream effectors of Akt are involved in varied aspects of cellular regulation, suggesting that Akt is a multifunctional protein kinase rather than merely a regulator of cell proliferation. Although, hyperplasia of colonic M-SMCs is common in IBD patients, their proliferation rate was not increased *in vitro* by poly I:C treatment. However, it has been reported that poly I:C treatment increases the proliferation of isolated human coronary artery SMCs 2–3-fold (Yang and others 2006).

This study explores the role that the PI3K/Akt pathway may play in the colonic M-SMC production of HA-cable-like polymers that are adhesive for mononuclear leukocytes. If the PI3K/Akt pathway is truly integral to the M-SMCs' ability to produce leukocyte adhesive HA, then inhibition of this signaling pathway should inhibit cable formation and leukocyte binding. Thus, M-SMC cultures were treated either without or with poly I:C in the absence or presence of LY294002. HA-cable-like structure formation as well as cell adhesion was assayed in parallel M-SMC cultures and was examined by confocal microscopy after staining for HA and nuclei. While poly I:C induced monocyte adhesion to the unique HA structure in M-SMC, the response was substantially inhibited by pre- and cotreatment of the M-SMCs with LY294002 (Fig. 3a). Toll like receptor (TLR)3 and retinoic acid inducible gene (RIG-I) have been shown to be involved in antiviral responses and melanoma differentiation associated gene-5 (MDA5) is essential for poly I:C-inducible interferon- α production in mice (Alexopoulou and others 2001; Kato and others 2005; Schroder and Bowie 2005; Sen and Sarkar 2005; Meylan and others 2006; Kawai and Akira 2008; Takeuchi and Akira 2008). Since no differences in leukocyte adhesion were observed in poly I:C-treated colonic SMCs isolated from wild-type and TLR3 null mice (our unpublished observation), the poly I:C-induced HA-mediated adhesion of U937 cells to mouse colonic SMCs is TLR3 independent. Confocal microscopy revealed abrogation of HA cable formation in the cultures treated with poly I:C plus LY294002 (Fig. 3a). PI3K is activated by various growth factor receptor-mediated signaling cascades and acts as a second messenger and subsequently mediates the activation of the serine/threonine kinase Akt by stimulating Akt phosphorylation (Datta and others 1999; Brazil and others 2002; Cantley 2002; Downward 2004; Fujita and others 2002; Franke and others 1997, 2003). Akt phosphorylation in poly I:C-stressed M-SMC cultures requires PI3K activity, and HA

cable formation as well as leukocyte adhesion are both abrogated by PI3K blockade. This study in M-SMCs indicates an early association of the PI3K and STAT1 after poly I:C treatment. This implicates PI3K as an essential mediator of HA cable formation and function.

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Author Disclosure Statement

Authors have no ethical or financial conflicts of interest to disclose.

References

- Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. 2001. Recognition of double-stranded RNA and activation of NF- κ B by Toll-like receptor 3. *Nature* 413:732–738.
- Amaravadi R, Thompson CB. 2005. The survival kinases Akt and Pim as potential pharmacological targets. *J Clin Invest* 115:2618–2624.
- Bandyopadhyay SK, de la Motte CA, Kessler SP, Hascall VC, Hill DR, Strong SA. 2008. Hyaluronan-mediated leukocyte adhesion and dextran sulfate sodium-induced colitis are attenuated in the absence of signal transducer and activator of transcription 1. *Am J Pathol* 173:1361–1368.
- Bandyopadhyay SK, de la Motte CA, Williams BR. 2000. Induction of E-selectin expression by double-stranded RNA and TNF- α is attenuated in murine aortic endothelial cells derived from double-stranded RNA-activated kinase (PKR)-null mice. *J Immunol* 164:2077–2083.
- Brazil DP, Park J, Hemmings BA. 2002. PKB binding proteins. Getting in on the Akt. *Cell* 111:293–303.
- Cantley LC. 2002. The phosphoinositide 3-kinase pathway. *Science* 296:1655–1657.
- Darnell JE Jr., Kerr IM, Stark GR. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264:1415–1421.
- Datta SR, Brunet A, Greenberg ME. 1999. Cellular survival: a play in three Akts. *Genes Dev* 13:2905–2927.
- Decker T, Kovarik P. 2000. Serine phosphorylation of STATs. *Oncogene* 19:2628–2637.
- de la Motte CA, Hascall VC, Calabro A, Yen-Lieberman B, Strong SA. 1999. Mononuclear leukocytes preferentially bind via CD44 to hyaluronan on human intestinal mucosal smooth muscle cells after virus infection or treatment with poly(I:C). *J Biol Chem* 274:30747–30755.
- de la Motte CA, Hascall VC, Drazba J, Bandyopadhyay SK, Strong SA. 2003. Mononuclear leukocytes bind to specific hyaluronan structures on colon mucosal smooth muscle cells treated with polyinosinic acid:polycytidylic acid: inter-alpha-trypsin inhibitor is crucial to structure and function. *Am J Pathol* 163:121–133.
- Downward J. 2004. PI 3-kinase, Akt and cell survival. *Semin Cell Dev Biol* 15:177–182.
- Dudley AC, Thomas D, Best J, Jenkins A. 2004. The STATs in cell stress-type responses. *Cell Commun Signal* 2:8–12.
- Feinberg MW, Jain MK. 2005. Role of transforming growth factor-beta1/Smads in regulating vascular inflammation and atherogenesis. *Panminerva Med* 47:169–186.

- Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C. 2003. PI3K/Akt and apoptosis: size matters. *Oncogene* 22:8983–8998.
- Franke TF, Kaplan DR, Cantley LC. 1997. PI3K: downstream AKTion blocks apoptosis. *Cell* 88:435–437.
- Fujita Y, Kitagawa M, Nakamura S, Azuma K, Ishii G, Higashi M, Kishi H, Hiwasa T, Koda K, Nakajima N, Harigaya K. 2002. CD44 signaling through focal adhesion kinase and its anti-apoptotic effect. *FEBS Lett* 528:101–108.
- Hu P, Han Z, Couvillon AD, Exton JH. 2004. Critical role of endogenous Akt/IAPs and MEK1/ERK pathways in counteracting endoplasmic reticulum stress-induced cell death. *J Biol Chem* 279:49420–49429.
- Kaser A, Blumberg RS. 2009. Endoplasmic reticulum stress in the intestinal epithelium and inflammatory bowel disease. *Semin Immunol* 21:156–163.
- Kaser A, Lee AH, Franke A, Glickman JN, Zeissig S, Tilg H, Nieuwenhuis EE, Higgins DE, Schreiber S, Glimcher LH, Blumberg RS. 2008. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell* 134:743–756.
- Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23:19–28.
- Kawai T, Akira S. 2008. Toll-like receptor and RIG-I-like receptor signaling. *Ann N Y Acad Sci* 1143:1–20.
- Kovarik P, Stoiber D, Eysers PA, Menghini R, Neiningner A, Gaestel M, Cohen P, Decker T. 1999. Stress-induced phosphorylation of STAT1 at Ser727 requires p38 mitogen-activated protein kinase whereas IFN- γ uses a different signaling pathway. *Proc Natl Acad Sci U S A* 96:13956–13961.
- Lin WW, Karin M. 2007. A cytokine-mediated link between innate immunity, inflammation, and cancer. *J Clin Invest* 117:1175–1183.
- Majors AK, Austin RC, de la Motte CA, Pyeritz RE, Hascall VC, Kessler SP, Sen G, Strong SA. 2003. Endoplasmic reticulum stress induces hyaluronan deposition and leukocyte adhesion. *J Biol Chem* 278:47223–47231.
- Meylan E, Tschopp J, Karin M. 2006. Intracellular pattern recognition receptors in the host response. *Nature* 442:39–44.
- Nguyen H, Ramana CV, Bayes J, Stark GR. 2001. Roles of phosphatidylinositol 3-kinase in interferon- γ -dependent phosphorylation of STAT1 on serine 727 and activation of gene expression. *J Biol Chem* 276:33361–33368.
- Puri KD, Doggett TA, Douangpanya J, Hou Y, Tino WT, Wilson T, Graf T, Clayton E, Turner M, Hayflick JS, Diacovo TG. 2004. Mechanisms and implications of phosphoinositide 3-kinase delta in promoting neutrophil trafficking into inflamed tissue. *Blood* 103:3448–3456.
- Schroder M, Bowie AG. 2005. TLR3 in antiviral immunity: key player or bystander? *Trends Immunol* 26:462–468.
- Sen GC, Sarkar SN. 2005. Transcriptional signaling by double-stranded RNA: role of TLR3. *Cytokine Growth Factor Rev* 16:1–14.
- Shiojima I, Walsh K. 2002. Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res* 90:1243–1250.
- Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. 1998. How cells respond to interferons. *Annu Rev Biochem* 67:227–264.
- Stephanou A, Scarabelli TM, Brar BK, Nakanishi Y, Matsumura M, Knight RA, Latchman DS. 2001. Induction of apoptosis and Fas receptor/Fas ligand expression by ischemia/reperfusion in cardiac myocytes requires serine 727 of the STAT-1 transcription factor but not tyrosine 701. *J Biol Chem* 276:28340–28347.
- Takeuchi O, Akira S. 2008. MDA5/RIG-I and virus recognition. *Curr Opin Immunol* 20:17–22.
- Tessner TG, Muhale F, Riehl TE, Anant S, Stenson WF. 2004. Prostaglandin E2 reduces radiation-induced epithelial apoptosis through a mechanism involving AKT activation and bax translocation. *J Clin Invest* 114:1676–1685.
- Vantler M, Caglayan E, Zimmermann WH, Baumer AT, Rosenkranz S. 2005. Systematic evaluation of anti-apoptotic growth factor signaling in vascular smooth muscle cells. Only phosphatidylinositol 3'-kinase is important. *J Biol Chem* 280:14168–14176.
- Yang X, Murthy V, Schultz K, Tatro JB, Fitzgerald KA, Beasley D. 2006. Toll-like receptor 3 signaling evokes a proinflammatory and proliferative phenotype in human vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 291: H2334–H2343.

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