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Functional Expression of Double-Stranded RNA-Dependent Protein Kinase in Rat Intestinal Epithelial Cells

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ABSTRACT

Intestinal epithelial cells (IECs) are exposed to external environment, microbial and viral products, and serve as essential barriers to antigens. Recent studies have shown that IECs express Toll-like receptors (TLRs) and respond to microbial components. The antimicrobial and antiviral barriers consist of many molecules including TLRs. To investigate the further component of this barrier in intestine, we examined the expression of double-stranded RNA-dependent protein kinase (PKR). PKR is a player in the cellular antiviral response and phosphorylates α-subunit of the eukaryotic translation initiation factor 2 (eIF-2α) to block protein synthesis and induces apoptosis. In this study, we showed that the expression of PKR was restricted to the cytoplasm of absorptive epithelial cells in the intestine of adult rat. We also demonstrated that PKR was expressed in the cultured rat intestinal epithelial cells (IEC-6). The level of PKR protein expression and the activity of alkaline phosphatase (ALP) increased in the cultured IEC-6 cells in a time-dependent manner. Inhibition of PKR by the 2-aminopurine treatment decreased ALP activity in the IEC-6 cells. Treatment of IEC-6 cells with synthetic double-stranded RNA (dsRNA) induced cell death in a dose-dependent manner. The addition of hydrocortisone also provoked suppression of PKR expression and ALP activity. This modulation might be mediated by signal transducers and activators of transcription-1 (STAT-1) protein. We concluded that PKR is expressed in IECs as potent barriers to antigens and is a possible modulator of the differentiation of rat IECs. J. Cell. Biochem. 110: 104–111, 2010.

KEY WORDS: PKR; STAT-1; DSRNA; INTESTINAL EPITHELIAL CELL; ALKALINE PHOSPHATASE ACTIVITY

The epithelium of the digestive tract is an important area for absorption of nutrients and innate immune system. The epithelium of the crypts and the villi in small intestine is morphologically divided into two cell populations based on the state of cellular proliferation and differentiation. The crypts contain immature proliferating stem cells and mitotically active progenitors, while intestinal epithelial cells (IECs) of the villi are mitotically inactive and terminally differentiated. IECs are constantly exposed to microbial and viral products and serve as essential barriers to these antigens. Recent studies have shown that IECs express Toll-like receptors (TLRs) that respond to microbial components [Abreu et al., 2005]. In addition to TLRs, many other molecules are responsible for a host defence system.

Double-stranded RNA-dependent protein kinase (PKR) is one of the main players in the cellular antiviral responses. PKR is a serine/threonine protein kinase expressed in mammalian cells [Hovanessian, 1989] and activated by double-stranded RNA (dsRNA), interferons (INFs), cytokines, stress signals, and viral infections [Williams, 1997; Sadler and Williams, 2008]. PKR becomes activated through autophosphorylation. Once activated, the enzyme phosphorylates certain substrates including α-subunit of eukaryotic translation initiation factor 2 (eIF-2α). Phosphorylation of eIF-2α results in the inhibition of protein synthesis followed by apoptosis [de Haro et al., 1996; Morimoto et al., 2004].

PKR is critical for many aspects of virus infections. For example, the activity of PKR kinase is inhibited by binding of hepatitis C virus

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(HCV) E2 envelope protein to PKR. The interaction gives the resistance to HCV against INFs [Taylor et al., 1999]. Focused into the intestine, rotavirus (dsRNA virus) infection may play an important role in stimulating PKR gene expressions in IECs [Vijay-Kumar et al., 2005]. Epstein–Barr virus-encoding small nonpolyadenylated RNA (EBER) confers resistance to the Fas-mediated apoptosis by blocking PKR activity in an intestinal cell line [Nanbo et al., 2005]. PKR-mediated detection of RNA in the epithelium is likely to play a broad role in recognizing intestinal viruses.

Several reports showed that PKR is aberrantly expressed in tumor cells, including carcinomas of lung [Haines et al., 1992], skin [Haines et al., 1998], colon [Singh et al., 1995], breast [Kim et al., 2000; Nussbaum et al., 2003], and liver [Shimada et al., 1998], and certain types of leukemias [Beretta et al., 1996; Basu et al., 1997]. The expression of PKR seems to depend on the differentiation stages of the tumors [Haines et al., 1992; Singh et al., 1995]. These findings suggest that the expression of PKR has a role in maintenance of tumor differentiation and progression. However, it is still unknown about the roles of PKR in normal digestive tract including small intestine. In the present study, we tried to clarify the relationship between PKR expression and differentiation of IECs.

Rat intestinal epithelial cell line (IEC-6) was established from crypt of rat intestinal cells and share many undifferentiated characters of the immature intestinal cells [Quaroni et al., 1979]. IEC-6 cells cultured for postconfluent or cultured on the extracellular matrix gel (Matrigel®) developed differentiation such as alterations of morphology and increased alkaline phosphatase (ALP) activity [Wood et al., 2003]. Glucocorticoids, another well-characterized maturation agent for IECs, induced cell-cycle arrest and caused morphological changes in IEC-6 cells. However, glucocorticoid treatment resulted in the decreased expressions of ALP, transforming growth factor (TGF)-β1 and -β2, and interleukin (IL)-1β [Quaroni et al., 1999; Schaeffer et al., 2000]. It is reported that circulating glucocorticoids are increased during the third week of postnatal life of rats. The increment is associated with the intestinal functions necessary to digest the adult diet [Schaeffer et al., 2000]. It is well known that the absorptive system in small IECs is dramatically changed during the weaning process [Baba et al., 2005; Fujita et al., 2007].

In this study, we examined the expression of PKR in rat small intestine, and evaluated the expression of PKR and ALP activity in IEC cell line, as well as an effect of glucocorticoid treatment of the cells.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's minimal essential medium (D-MEM) and recombinant insulin were purchased from Gibco BRL (Gaithersburg, MD). Penicillin–streptomycin mixture and 2-aminopurine (2-AP) were purchased from Sigma–Aldrich (St. Louis, MO). Cell culture plastic dishes were from IWAKI Glass (Chiba, Japan) and fetal bovine serum (FBS) from JRH Biosciences (Lenexa, KS). The following antibodies were used: anti-PKR (YE350; Abcam, Cambridge, MA), anti-β-actin (Sigma–Aldrich), anti-STAT-1 (Cell Signalling, Danvers, MA), and antiproliferating cell nuclear antigen (PCNA) (Dako, Glostrup, Denmark). Poly (I:C) was purchased from Invivogen (San Diego, CA). Other materials used were of the highest grade commercially available.

ANIMALS

Eight-week-old Brown Norway rats were used in the present study. The protocol for the research was approved by a suitably constituted ethics committee of the institution within which the work was undertaken, and it conformed to the provision of the Declaration of Helsinki, 1995 (as revised in Edinburgh, 2000). In all the experiments, the animals were anesthetized with ether. The small intestine was exposed through a vertical slit in the abdominal wall and the entire small intestine was dissected intact. The proximal jejunum was cut into 5 mm rings.

RNA EXTRACTION AND REAL-TIME PCR ANALYSIS

Total RNA was isolated from rat small intestine using ISOGEN (Nippongene, Tokyo, Japan). One microgram of total RNA was used for first-strand cDNA synthesis, using superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA) and oligo (dT) primer according to the manufacturer's protocol. One microliter of the cDNA solution was used as a template for each reaction of PCR. PCR was performed on the cDNA with high fidelity polymerase (pfx DNA polymerase, Invitrogen) by using the following primers: PKR, Fwd: 5′-TGGTGACCAGGGTCTATGCTTTC-3′, Rev: 5′-GATCATCTG-CCCACCCTGCTA-3′; β-actin, Fwd: 5′-TCATGAAGTGTGACGTT-GACATCCGT-3′, Rev: 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′; Amplification products were separated on 2% agarose gel, stained with ethidium bromide, and visualized with an ultraviolet transilluminator.

PREPARATION OF TISSUE

For light microscopic study, the specimens were fixed in 4.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for overnight at 4°C, washed in the same buffer, dehydrated in a graded series of ethanol before being replaced by xylene, and embedded in paraffin wax (Merck, Darmstadt, Germany). Thin sections were cut into 5-μm slices, processed for immunohistochemistry.

IMMUNOHISTOCHEMISTRY

For detection of PKR, deparaffinized sections were immersed in 20 mM glycine–HCl buffer (pH 3.0) with using microwave at 800 Ws (MICROMED T/T, Milestone, Sorisole, Italy) for 20 min. Antigen-retrieved sections were blocked with 1.0% bovine serum albumin (BSA)–phosphate-buffered saline (PBS) for 1 h at room temperature. To detect the localization of PKR and PCNA, the rabbit monoclonal anti-PKR antibody and mouse monoclonal anti-PCNA antibody were used as primary antibodies. The sections were incubated with the primary antibodies diluted 1:250 in 1.0% BSA–PBS for overnight at 4°C. After being washed three times with PBS, all sections were incubated with secondary antibodies diluted 1:500 in 1.0% BSA–PBS for 45 min at room temperature. As secondary antibodies, Alexa Fluor 488-labeled goat anti-rabbit IgG and Alexa Fluor 546-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR) were used. All sections were washed three times with PBS, mounted with a Vectashield Hard Set Mounting Medium (Vector

Laboratories, Burlingame, CA) and observed with a fluorescence microscope (Axioskop 2 plus, Carl Zeiss, Göttingen, Germany). Specificity of immunoreactivity was confirmed by replacing the primary antibodies with either normal sera or 1.0% BSA–PBS.

CELL CULTURE

IEC-6 cells were obtained from RIKEN CELL BANK (Ibaraki, Japan) and were cultured in plastic dishes containing D-MEM supplemented with 5% (v/v) FBS, 4 μg/ml insulin, 10 μg/ml penicillin, and 10 μg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every 3 days. In the treatment of glucocorticoid or 2-AP, subconfluent cells were cultured in basic medium containing 500 nM hydrocortisone (Biochrom AG, Berlin, Germany) or 2-AP at various concentrations. For immunocytochemical study, the cells were cultured on the coverslips, after fixation in 10% formalin for 10 min, the cells were permeabilized in methanol for 20 min at −20°C.

SDS–PAGE AND WESTERN BLOTTING

The specimens and cells were washed twice with Ca^{2+}-, Mg^{2+}-free PBS(−) and then lysed into lysate buffer containing protease inhibitor cocktail (complete EDTA-free, Roche, Basel, Switzerland) in PBS. The cells were sonicated for a few seconds using a sonifier-cell disruptor (Homogenizer Subsonic HOM-100, IWAKI Glass), and centrifuged at 13,200*g* for 20 min at 4°C. The concentration of the protein was determined using the Protein Assay Reagent (Bio-Rad, Hercules, CA). The samples were denatured in sodium dodecyl sulfate (SDS) sample buffer and heated in boiling water for 5 min. Equal amount of proteins and prestained molecular weight markers were separated by 10% SDS–PAGE and transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were incubated for 2 h at room temperature in a blocking solution consisting of 5% skim milk in PBS containing 0.1% Tween-20 (PBS-T), and incubated for overnight at 4°C with specific antibodies in PBS-T (diluted at 1:1,000). After the membranes had been washed four times within 30 min in PBS-T, they were incubated for 1 h at room temperature in PBS-T containing 80 ng/ml horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA). The membranes were washed in PBS-T, and the proteins recognized by the antibodies were detected with an ECL detection kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's directions. To strip off the antibodies for reuse of the membranes, we incubated the membranes for 30 min at 50°C with 2% SDS and 0.35% 2-mercaptoethanol in 62.5 mM Tris–HCl buffer (pH 6.8). The antibody stripped membranes were then blocked again and re-incubated with another type of antibody.

ALKALINE PHOSPHATASE ACTIVITY ASSAY

For assay of ALP activity, the cells were cultured for the desired time and thereafter washed in physiological saline. Extraction solution (physiological saline containing 1% NP-40) was added to each cell group and was pipetted gently to lyse the cells. The ALP activity in the supernatant was then determined using *p*-nitrophenyl phosphate (*p*NPP) as a substrate according to the method of ALP Assay Kit (Takara Bio, Otsu, Japan). The substrate solution was added and reacted for 60 min at 37°C. As an endpoint assay, we stopped the reaction with 0.9 N NaOH and measured the absorbance at 405 nm. The experiments were done three times with three separate samples. The values (mean ± SEM) were presented as percentages of the control cultures. Data were analyzed by Student's *t*-test calculations by Microsoft Excel 2008 (Microsoft, Redmond, WA).

CELL VIABILITY MEASUREMENT BY WST-1 ASSAY

Viability of IEC-6 cells was measured by the Cell Proliferation Reagent WST-1 quantitative colorimetric assay (Roche) for cell survival. The assay detects living cells, and the signal generated is dependent on the degree of mitochondrial activity in the cells. Subconfluent cells grown in six-well culture plates were treated for 72 h with poly (I:C) and Fugene HD (Roche). The WST-1 solution (200 μl) was immediately added to a well containing 2 ml of culture medium, and the cells were then incubated for an additional 4 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After shaking thoroughly for 1 min on a shaker, the absorbance of the medium was measured at 420 nm for test samples and 600 nm as a reference with a microplate reader (Model 680, Bio-Rad). The experiments were done three times with three separate samples. The values (mean ± SEM) were presented as percentages of the control cultures.

RESULTS

DISTRIBUTION OF PKR IN SMALL INTESTINE OF RAT

PKR mRNA and protein expressions in small intestine of adult rat were examined by RT-PCR and Western blot analysis. PKR mRNA was detected as a 123 bp RT-PCR product in total RNA sample from rat small intestine (Fig. 1A). RT(−) samples and β-actin primers were used for negative- and positive-control for the reaction, respectively. Immunoblot analysis revealed that the anti-PKR antibody specifically reacted with a 58 kDa band in the extracts from rat small intestine (Fig. 1B). A band corresponding to the position of PKR was not detected in the blots of the extracts incubated with the same dilution of normal rabbit serum (data not shown).

To determine the distribution of PKR in small intestine of rat, we performed immunohistochemical analysis on intestinal sections. As seen in Figure 2a, PKR was expressed in the absorptive cells of entire villi but not in the epithelial cells of the crypts. PKR localized in the cytoplasm of the cells. These results indicated that PKR was expressed in rat small intestine and could be detected by using the rabbit monoclonal antibody. The preimmune serum did not show any staining at the same dilution (data not shown). The distribution of PCNA, which is known to localize in the proliferating cell nuclei, was also examined immunohistochemically. PCNA was detected in the nuclei of the epithelial cells in the crypts, in which the cells have been shown to be actively proliferating (Fig. 2b). The merged view indicates that the distribution of PKR and PCNA are completely inverse (Fig. 2c).

EXPRESSION OF PKR IN THE CULTURED RAT IECS (IEC-6)

To examine the expression of PKR in the cultured IEC-6 cells, we fixed, permeabilized, and stained the IEC-6 cells with anti-PKR antibody (Fig. 3a). The nuclei in the same cells were stained with

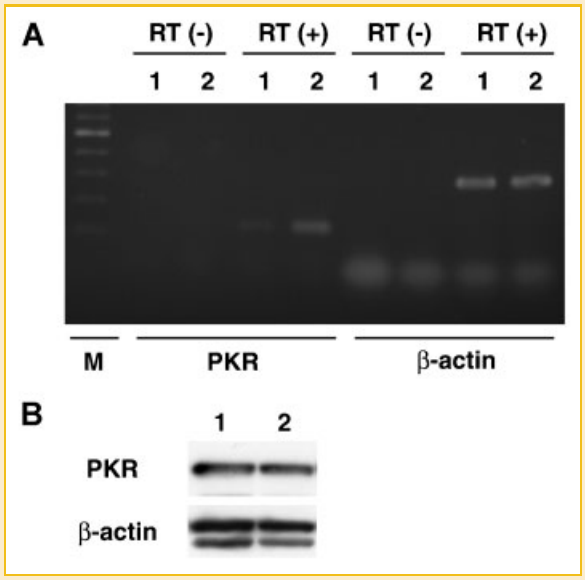

Fig. 1. Identification of mRNAs and proteins of PKR in small intestine of adult rat. A: Total RNA was prepared from small intestine and the cDNA from the mRNA was amplified with PCR. The resulting products of RT-PCR were electrophoresed in 2% agarose gels and stained with ethidium bromide. Lane M: standard DNA marker. RT (−), PCR product from RNAs without reverse transcription. These experiments were done with two separate rats (lanes 1 and 2). B: Tissue extracts prepared from small intestine were analyzed by Western blotting. The experiments were done with two separate rats (lanes 1 and 2). The antibody was stripped off the membrane, which was re-incubated with anti-β-actin antibody as a loading control.

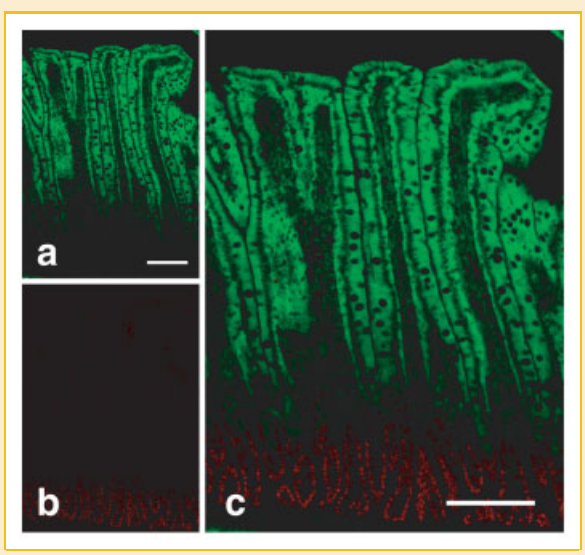

Fig. 2. PKR expression in intestine of adult rat. Immunohistochemical analysis was performed on 5-μm thick paraffin-embedded sections of small intestine of adult rat. Positive staining was seen as a green (PKR) and red (PCNA). a: PKR, (b) PCNA, and (c) merged view of (a) and (b). Bars represent 100 μm.

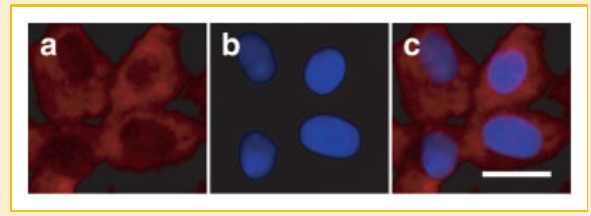

Fig. 3. Subcellular localization of PKR in intestinal epithelial cell line IEC-6 cells. Immunocytochemical analysis was performed on IEC-6 cells cultured on the coverslips. The cells were stained with anti-PKR antibody (red) and DAPI (blue). PKR exists in the entire cytoplasm. a: PKR, (b) DAPI, (c) merged view of (a) and (b). Bar represents 30 μm.

DAPI as shown in Figure 3b. Merged view indicates that PKR is detected predominantly in cytoplasm of the cultured cells (Fig. 3c). We also performed Western analysis of proteins in IEC-6 cells. As shown in the upper panel of Figure 4, a 58 kDa band corresponding to the estimated molecular weight of PKR was detected in the cell extracts of the cultured IEC-6 cells. The expression of PKR protein increased in a time-dependent manner. The level of β-actin did not change in these cells (Fig. 4, middle panel). The level of PKR expression at longer than 5 days of postconfluence increased threefold over than that of the confluent stages (Fig. 4, bottom panel).

EFFECTS OF PKR INHIBITOR ON ALP ACTIVITY IN THE IEC-6 CELLS

To reveal the statues of the differentiation of IEC-6 cells and the effect of the 2-AP treatment on the differentiation of the cells, we examined the ALP activity in these cells. The IEC-6 cells were cultured with or without 2-AP, and the ALP activity was measured at times indicated in Figure 5. ALP activity in IEC-6 cells increased as culture time proceeded (Fig. 5A). After the 3-day cultivation, the ALP activity increased more than eightfold that of the cells at the confluent stage. The ALP activity was quite low in the 2-AP-treated cells during the entire culture periods (Fig. 5B). At day 3, the ALP activity in the untreated cells was tenfold higher than that of the 2.5 mM 2-AP-treated cells.

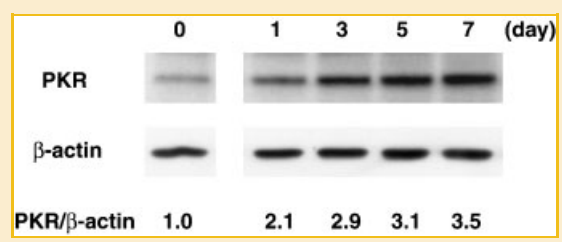

Fig. 4. PKR expression in IEC-6 cells. The cells were cultured for the indicated periods and cell lysates were prepared. Twelve micrograms of proteins of each sample were separated on a 10.0% of SDS–PAGE, transferred to PVDF membrane, and incubated with anti-PKR antibody (upper panel). The antibody was stripped off the membrane, which was then re-incubated with anti-β-actin antibody as a loading control (middle panel). The intensity of the immunopositive bands from the blot was quantitated (bottom panel).

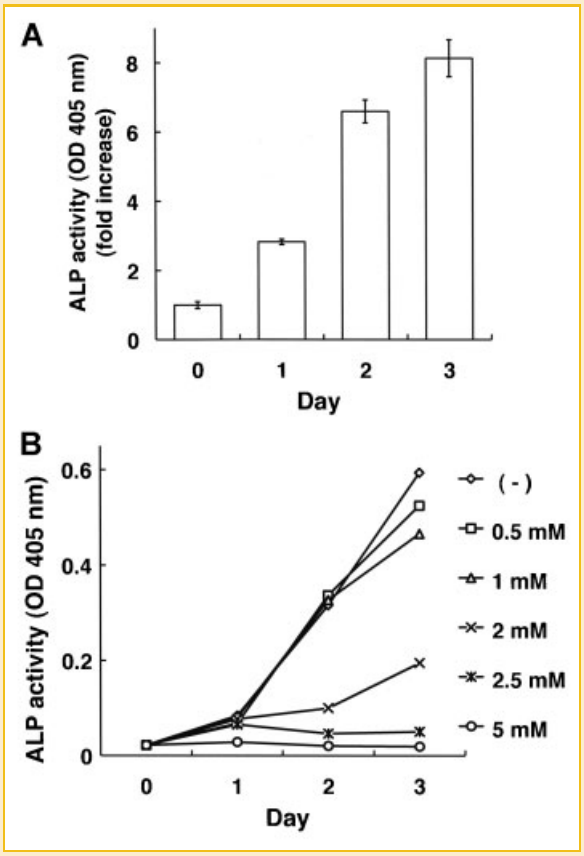

Fig. 5. ALP activity in IEC-6 cells. A: ALP activity was measured in the cultured IEC-6 cells at days indicated. Each plot shows the average of four different cultures, and the bars represent the SEM. The results are representative of three independent experiments. B: Suppression of ALP activity by PKR inhibitor. IEC-6 cells were treated with various concentrations of 2-AP, and ALP activity was measured in the cells at days indicated.

EFFECTS OF DOUBLE-STRANDED RNA ON IEC-6 CELLS

To assay the functional significance of the PKR expression, we treated IEC-6 cells with synthetic dsRNA at various concentrations for 48 h. Treatment of IEC-6 cells with poly (I:C) caused the decreased viability of the cells in a dose-dependent manner (Fig. 6). The mitochondrial oxidative activity measured by the WST-1 assay in the 0.1 μg poly (I:C)-treated cells was 74% that of the untreated control cells. However, the viability of IEC-6 cells treated with a higher concentration of poly (I:C) (3 μg) was only 10% that of the untreated control cells.

EFFECTS OF HYDROCORTISONE ON ALP ACTIVITY AND THE EXPRESSION OF PKR AND STAT-1

When IEC-6 cells were treated with 500 nM hydrocortisone, ALP activity was suppressed (Fig. 7A, solid bars) compared with the activity of the untreated culture (Fig. 7A, open bars). At day 5, the ALP activity in the untreated cells was twofold higher than that of the hydrocortisone-treated cells. As already shown in Figure 4, PKR expression in IEC-6 cells increased as culture periods prolonged. The ratio of PKR expression to β-actin increased up to 3.1 and 3.5 at the 5- and 7-day culture, respectively, compared

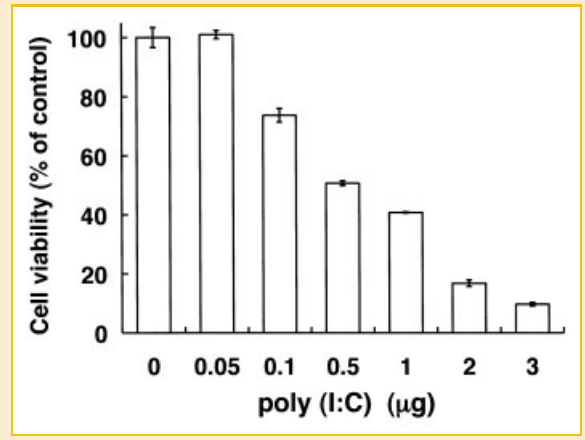

Fig. 6. Effects of poly (I:C) on cytotoxity in IEC-6 cells. Cells grown in six-well plates were cultured for 72 h with various concentrations of poly (I:C). The number of living cells was determined using the WST-1 assay. The activity was compared to the control and results are expressed as a percentage of the control (means ± SEM) (n = 3). The results are representative of three independent experiments.

with that of the confluent stage of IEC-6 cells. However, the ratio in the cells treated with hydrocortisone was only 1.9 and 2.4, respectively (Fig. 7B). From the densitometric analysis, the ratio of PKR expression to β-actin in the cells treated for 5 and 7 days with hydrocortisone was 61% and 69%, respectively, that of the untreated cells (Figs. 4 and 7). These results indicate that hydrocortisone-treatment suppressed PKR expression in IEC-6 cells (Fig. 7B). The level of STAT-1 protein in the IEC-6 cells increased as culture time proceeded, whereas the hydrocortisone-treatment suppressed the expression of STAT-1 in the cultured IEC-6 cells (Fig. 8). Densitometric analysis revealed that STAT-1 expression in the hydrocortisone-treated cells (HYD (+)) was almost half of the untreated control cells (HYD (−)).

DISCUSSION

In this study, we examined the expression and localization of PKR in small intestine of adult rat. PKR localized in the cytoplasm of IECs in the entire villi. PKR did not co-localize with PCNA. We also demonstrated that PKR was expressed in the cultured rat IEC line, IEC-6 cells. The level of PKR protein expression and ALP activity increased in continuous postconfluent cultures.

It has been shown that PKR is a key molecule in a wide range of cellular processes [García et al., 2006]. PKR regulates apoptosis, cell proliferation, signal transduction, and differentiation [Morimoto et al., 2004, 2005; Yoshida et al., 2005]. PKR protein was constitutively expressed in keratinocytes of normal skin and mucosa. These results indicated that a loss of PKR was associated with increase of cellular proliferative activity and alteration of cellular differentiation [Kuyama et al., 2003]. PKR expression decreased in keratinocytic tumor, whereas PCNA expression increased in these cells. Similar inverse distribution of PKR and PCNA was detected in squamous cell carcinoma cells [Haines et al., 1998; Kuyama et al., 2003]. However, it was reported that an

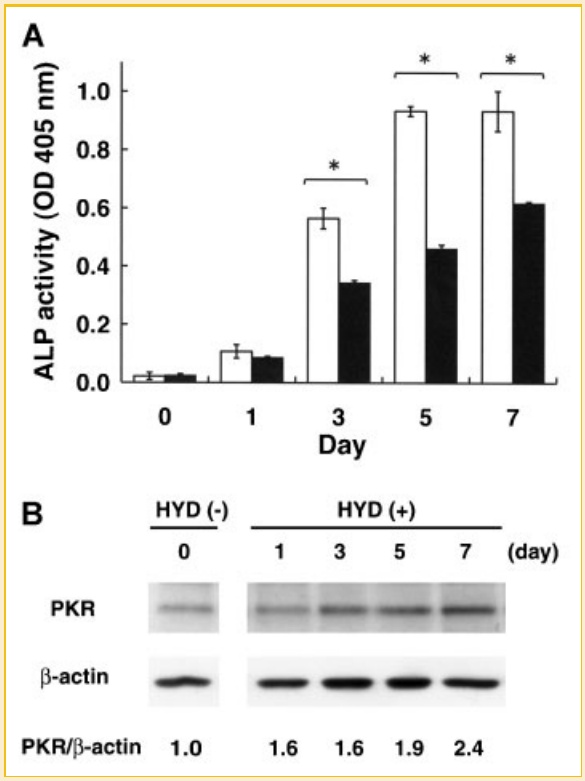

Fig. 7. Effects of hydrocortisone on IEC-6 cells. A: ALP activity was measured in the hydrocortisone-treated (solid bars) or -untreated (open bars) cells at the days indicated. Each plot shows the average of three different cultures, and the bars represent the SEM. The results are representative of three independent experiments. Significant difference are indicated by an asterisk, $^{*}P < 0.01$ (Student's *t*-test). B: Western blotting analysis on the hydrocortisone-treated IEC-6 cells. Hydrocortisone-treated [HYD (+)] or -untreated [HYD (−)] cells were cultured for the indicated periods, and the cell lysates were prepared. Twelve micrograms of proteins of each sample were separated on a 10.0% of SDS–PAGE, transferred to PVDF membrane, and incubated with anti-PKR antibody. The antibody was stripped off the membrane, which was then re-incubated with anti-β-actin antibody as a loading control. The intensity of the immunopositive bands from the blot was quantified.

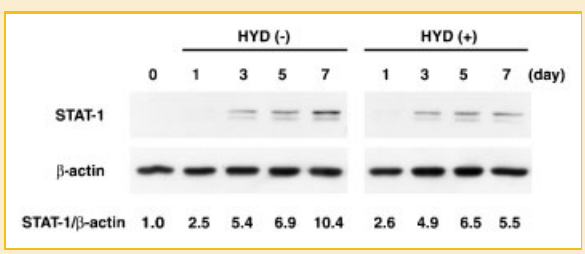

Fig. 8. Modulation of STAT-1 protein expression in the hydrocortisone-treated IEC-6 cells. Western blot analysis on the hydrocortisone-treated IEC-6 cells. The cells were treated with hydrocortisone for various time periods and the samples were analyzed by the Western blotting with anti-STAT-1 antibody. The stripped membrane was re-used for staining with anti-β-actin antibody as a loading control. The intensity of the immunopositive bands from the blot was quantified.

expression of PKR was observed in the crypts of normal colonic mucosa which express PCNA [Kim et al., 2002]. Although the detailed reason for this variance is not clear, there are significant differences in the morphological features and the functions between the epithelium of colon and small intestine. The expression of PKR in small intestine might not be the same as that in colon. Indeed, in the present study, we demonstrated that PKR was expressed in mitotically inactive, terminally differentiated epithelial cells of the villi and the expression of PCNA was restricted in the crypts where the immature cells proliferate. This inverse distribution is the same as the previous reports [Haines et al., 1998; Kuyama et al., 2003]. PKR regulates cell differentiation to different lineages. In the PKR-dominant negative cells, inhibited endogenous PKR activity interferes with myogenesis of murine C2C12 cells [Salzberg et al., 2000] and calcification of osteoblast [Yoshida et al., 2005]. The PKR expression and ALP activity were upregulated after postconfluent cultures, whereas 2-AP treatment suppressed the increment of ALP activity. The similar result was obtained by another specific inhibitor of PKR (PKR inhibitor, Calbiochem) (data not shown). These results suggest that the expression of PKR and its activity play a role in the modulation of cell growth and differentiation.

Glucocorticoids (e.g., hydrocortisone and dexamethasone) are major immune suppressive and antiinflammatory factors. Although the PKR expression was upregulated after postconfluent cultures, hydrocortisone-treatment suppressed the increment of PKR expression and ALP activity in the treated cells. Another type of immune suppressive reagent, calcineurin inhibitor Tacrolimus, also had an inhibitory effect on INF-α-induced PKR expression [Hirano et al., 2008]. These modulations may be partially mediated by STAT-1 protein. STAT-1 is constitutively expressed in many types of cells and its expression is induced by type I and type II INFs and other factors. IFN-γ-activated-STAT-1 expression was inhibited by the dexamethasone treatment in the cultured peripheral blood mononuclear cells [Hu et al., 2003]. Furthermore, glucocorticoids suppress the expression of STAT-1 and diminish the signaling of INFs. Downregulation of STAT-1 is another possible mechanism underlying the suppression of IFN-γ signaling. Major immune suppressive effect of glucocorticoid may be derived from the inhibition of NF-κB and AP-1 transcriptional activities and thus suppress the expression of inflammatory cytokines, such as IL-1 and TNF. In our study, the suppression of PKR by hydrocortisone should be mediated by STAT-1/IFN-γ mechanism, because PKR is an IFN inducible protein.

PKR is activated during infection and various cellular stresses and is identified as an important component of the pro-inflammatory response [Gusella et al., 1995; Williams, 1999]. Treatment of IEC-6 cells with poly (I:C) induced cell death at the concentration of 0.1 μg. This result indicates that PKR expressed in IEC-6 cells recognizes dsRNA and induces cell death. PKR is essential for the dsRNA response in type I INF induction and the induction of apoptosis by both polymerase-synthesized RNAs and poly (I:C) [Kalali et al., 2008; McAllister and Samuel, 2009].

There are no apparent abnormalities on the formation of intestine in $PKR^{-/-}$ mice [Yang et al., 1995]. However, our results showed that PKR expressed in rat IECs in vivo and in vitro may recognize dsRNA

and play an important role in antiviral function. We predict the functional disabilities of intestine in these mice.

In conclusion, the data presented in this study indicates that the kinase PKR is expressed during the differentiation of IECs. Our results suggest that PKR plays key roles in regulation of intestinal innate immune defence system. We also demonstrated that the suppression of PKR expression with hydrocortisone treatment, partially mediated by STAT-1, was one of the possible underlying mechanisms of immune suppressive reagents. Our results will contribute to a better understanding of normal development of intestine and intestinal innate immune system. It is important to understand how PKR is regulated during cell growth and differentiation.

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