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*J Immunol* 2000; 164:1078-1085; 
doi: 10.4049/jimmunol.164.2.1078

http://www.jimmunol.org/content/164/2/1078

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Decay-Accelerating Factor in Guinea Pig Stomachs Following Ischemia Reperfusion Stress

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A complement regulatory protein, decay-accelerating factor (DAF, CD55), is known to protect host tissues from autologous complement activation. DAF is present on the apical side of human gastric epithelial cells, and its expression increases during gastritis. To develop an animal model for analysis of DAF expression on gastric cells, a mAb to guinea pig DAF was successfully used. Although DAF expression in the mucosal epithelium of the stomach is weak, as judged by immunohistochemical staining with the mAb, it was temporarily up-regulated at 12 and 24 h, and at 3 days after ischemia reperfusion (I/R) (p < 0.05). The DAF mRNA level in gastric tissues was determined by Northern blot analysis and found to be highest at 6 h after I/R, returning to the baseline at 24 h. Strong DAF mRNA expression was observed in the cytoplasm of cells beneath the eroded tissues 6 h after I/R. In guinea pigs, alternative splicing of DAF mRNA generates both GPI-anchored types and transmembrane types of DAF. RT-PCR analysis revealed that mRNAs of the transmembrane types had become significantly dominant by 6 h after I/R, whereas levels for the GPI-anchored types remained unchanged. In guinea pigs depleted of complement by cobra venom factor treatment, the area of erosion and the up-regulation of DAF expression in gastric epithelial cells after I/R were significantly limited compared with the normocomplementemic group, indicating that DAF may be up-regulated by an inflammatory stress.


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Materials and Methods

Animal preparation

Male Hartley GPs weighing 250–300 g (Chubu Kagaku Shizai, Nagoya, Japan), were subjected to an overnight fast. After induction of anesthesia with pentobarbital sodium (30 mg/kg, i.p. administration), animals were placed on an electric heating pad in the supine position. Body temperature was maintained at 37°C throughout the experiment. Gastric ischemia was induced by clamping the left gastric and gastric epiploic arteries for 30 min. Before sacrifice, stomachs were removed at 0, 6, 12 and 24 h, and at 3 and 6 days after I/R, then washed with 0.9% NaCl.

In another series of experiments, GPs were divided into two groups: a group that was i.p. administered saline (−CVF group), and another that received 50 units of CVF that were i.p. injected 24 h before the experiment to deplete complement (+CVF group).

CVF

CVF was purified on DEAE cellulose from lyophilized cobra venom (Wako Pure Chemical Industries, Tokyo, Japan) and was further purified.
on a Mono Q column (Pharmacia Biotech, Tokyo, Japan) to remove phospholipase A. In GPs i.p. injected with 50 units of purified CVF, the complement activity as measured by the CH50 level had become undetectable by 24 h after injection.

**Histology and immunohistochemistry**

The stomach was cut open along the greater curvature, pinned out on a cardboard, and fixed with acetic at 4°C for 3 days. The presence and severity of mucosal epithelial injury was then evaluated by macroscopic and microscopic assessment. The area of macroscopic hemorrhage and erosion was assessed by planimetry (NIH image). The lesion index is the percentile of the lesion area to the total area of stomach. Two strips of tissue were removed from the most eroded part of the stomach of five animals at all time points and embedded in paraffin. Histological change was assessed on sections stained with hematoxylin and eosin.

Immunoreactivity was demonstrated with the streptavidin-biotin complex (sABC) method using a HISTFINE SAB-PO (M) kit (Nichirei, Tokyo, Japan). Paraffin sections were cut to a thickness of 5 μm, deparaffinized with xylene, and dehydrated with acetone. After washing with PBS, the sections were treated with 3% (v/v) hydrogen peroxide in a 1:10 dilution of nonimmune rabbit serum for 10 min to block nonspecific Ig binding sites. After bluing up the excess serum, incubation was conducted with a specific mAb for GP DAF (18) for 1 h at room temperature. Control sections were treated with PBS or nonimmune mouse IgG1 (Dako, Glostrup, Denmark). The sections were rinsed and incubated sequentially with biotin-labeled rabbit anti-mouse IgG Ab for 10 min. The sections were then washed with PBS, and incubation was performed with peroxidase-labeled streptavidin-biotin for 5 min. After the sections were washed with PBS, they were stained with 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) solution containing 0.03% (v/v) hydrogen peroxide and 10 mM sodium azide. The sections were then counterstained with 1 μg/ml methyl green solution, washed with PBS, dehydrated in graded concentrations of ethanol, and mounted on glass slides. DAF expression on epithelial cells was judged by a semiquantitative scoring system that included the number of positively stained epithelial cells as well as the staining intensity: 0 = no visible staining; 1 = moderate staining of a few cells with weak staining of surrounding cells; 2 = extensive staining of most cells in specific regions with weak staining of surrounding cells. Scoring was monitored by a second observer who had no knowledge of the experimental protocol.

**RNA extraction and Northern blot analysis**

Total RNA extracts were prepared from GP stomachs using the TRIZOL system (Life Technologies, Tokyo, Japan). Polyadenylated RNA was isolated as mRNA. Samples of mRNA (5 μg) were separated electrophoretically on a 1% agarose gel containing 0.66 M formaldehyde, and transferred to a hybond-N nylon membrane (Amersham Japan, Tokyo, Japan). The membranes were then rinsed and incubated sequentially with biotin-labeled rabbit anti-mouse IgG Ab for 10 min. The sections were then washed with PBS, and incubation was performed with peroxidase-labeled streptavidin-biotin for 5 min. After the sections were washed with PBS, they were stained with 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) solution containing 0.03% (v/v) hydrogen peroxide and 10 mM sodium azide. The sections were then counterstained with 1 μg/ml methyl green solution, washed with PBS, dehydrated in graded concentrations of ethanol, and mounted on glass slides. DAF expression on epithelial cells was judged by a semiquantitative scoring system that included the number of positively stained epithelial cells as well as the staining intensity: 0 = no visible staining; 1 = moderate staining of a few cells with weak staining of surrounding cells; 2 = extensive staining of most cells in specific regions with weak staining of surrounding cells. Scoring was monitored by a second observer who had no knowledge of the experimental protocol.

**RT-PCR**

The following oligonucleotides were synthesized and used as primers: P1, 5′-GACACCTAGAATAG-3′; P2, 5′-TGCGGACGACCTGATA CCA-3′; P3, 5′-ATCGGCTGTTCCCG-3′; and P4, 5′-CAGCTAGC CAATGATTA-3′. cDNAs were prepared from RNAs from stomachs using a CDNA Synthesis System (Amersham Japan). PCR amplification was performed at 95°C for 3 min, followed by 20–30 cycles of 95°C for 0.5 min, 42°C for 0.5 min, and 72°C for 1 min, and then at 72°C for the final 5 min. The PCR products were analyzed on a 2% agarose gel or a 6% polyacrylamide gel.

**In situ hybridization (ISH)**

Samples of total RNA (15 μg) were separated electrophoretically on a 1% agarose gel containing 0.66 M formaldehyde and transferred to charged nylon membranes, which were then baked for 2 h at 80°C and treated with prehybridization buffer containing 5× SSC, 10× Denhardt’s solution, 10 mM Na2HPO4 (pH 6.5), 0.5% SDS, 0.1 mg/ml denatured salmon sperm DNA, and 50% denatured formamide at 65°C for 4 h. An ~570-bp fragment of GP DAF cRNA corresponding to the SCR2-SCR4 region was labeled with digoxigenin. The blotted RNAs were hybridized with the antisense and sense RNA probes (1 μg/ml) at 65°C for 16 h. After two washings with 2× SSC containing 0.1% SDS for 15 min each, the membranes were visualized using the alkaline phosphatase-conjugated anti-digoxigenin Ab and the chemiluminescent detection method. The agarose gel was also stained with ethidium bromide for detection of ribosomal RNA before the transfer to the membrane.

For ISH, animals were anesthetized with pentobarbital and killed by transcardiac perfusion with PBS, followed by perfusion of 4% paraformaldehyde solution in 0.1 M phosphate buffer. Each stomach was divided into 5-mm-thick sections and postfixed with the same fixative for 5 h at 4°C, then dehydrated in an ascending series of ethanol and embedded in paraffin. Five-micrometer-thick sections were mounted on poly-L-lysine-coated glass slides and deparaffinized. Tissue sections were treated with proteinase K (10 μg/ml solution in 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA) for 10 min at 37°C, and treated with 0.1 M triethanolamine-HCl (pH 8.0) buffer containing 0.25% acetylene hydrate for 10 min at room temperature.

Prehybridization, hybridization, and posthybridization were conducted as described by Matsukawa et al. (20) with minor modifications. For prehybridization, the specimens were incubated for 1 h at 50°C in a solution containing 50% formamide, 2× SSC, 25 mM DTT, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA. Hybridization was performed for 18 h in a moist chamber at 50°C with 60 ng of antisense or sense riboprobe in a 150-μl hybridization solution. The nonhybridized probes were removed by consecutive washings at 50°C for 30 min with buffer containing 50% formamide, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 2× SSC, and treated at 37°C for 30 min with 20 μg/ml RNase A in a buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.5 M NaCl. For the detection of hybridization signals, the hybridized sections were treated with alkaline phosphatase-conjugated anti-digoxigenin Ab using high m.w. polyvinyl alcohol and subjected to the alkaline phosphatase indoxyl-nitroblue tetrazolium reaction. The specificity of the reaction was tested by incubation with the labeled sense probe, omission of the labeled anti-sense probe, and by competition between labeled and unlabeled antisense probes.

**Data analysis**

Dunnett’s post hoc procedure and Fisher’s protected least significant difference were used to compare the mean values among the groups. All data are expressed as means ± SE. Significance was accepted at p < 0.05.

**Results**

**Mucosal epithelial injury after I/R**

Lesion indices (see *Materials and Methods* section) at 0, 6, 12 and 24 h, and 3 and 6 days after I/R are shown in Fig. 1. Mucosal damage was significant from 6 h to 3 days after I/R (p < 0.05), but, at 6 days after I/R, no significant erosion remained. Significant neutrophil infiltration was observed around 6 h after I/R (Fig. 2B) but was only slight at the site of erosion at 0 h (Fig. 2A), 24 h (Fig. 2C), and 6 days (Fig. 2D).

**Changes in expression of DAF protein after I/R**

Result of immunohistochemistry staining performed with a mAb against GP DAF is shown in Fig. 3. Although DAF immunoreactivity was consistently observed on mucosal microvascular endothelial cells, only a minute amount of DAF immunoreactivity was observed in normal GP gastric epithelial cells (Fig. 3A). But a strong immunoreactivity was observed 24 h after I/R, predominantly on the luminal surface of epithelial cells (Fig. 3B, indicated by arrows). Only a slight immunoreactivity was detectable 6 days after I/R (Fig. 3E). No staining was observed at any time point with the control nonimmune mouse IgG1 (Fig. 3, B, D, and F).

Expression of DAF in GP gastric epithelial cells was semi-quantitatively scored into three grades and was statistically analyzed.
Although no significant change was observed until 6 h after I/R, a significant increase in DAF expression was found at 12 and 24 h, and at 3 days (\( p < 0.05 \)), after which DAF expression decreased to less than significant levels (Fig. 4).

Changes in expression of DAF mRNA after I/R

Northern blot analysis with 0.9 kb GP DAF cDNA corresponding to the SCR1-SCR4 region demonstrated two relatively broad bands of 2.4 to 2.5 kb and 1.6 to 1.8 kb. DAF mRNA was constitutively detected in normal gastric mucosa, and the amount was significantly increased at 6 h, returning to a normal level at 24 h (Fig. 5) (1 h, 149 ± 12% of control, \( p < 0.05 \); 6 h, 228 ± 20%, \( p < 0.001 \); 24 h, 105 ± 6%, NS).

Changes in the isoform expression of DAF mRNA were determined at 0 and 6 h after I/R. Fig. 6A shows the PCR products obtained with the region between the ST-d and G (19) regions (lanes 1 and 2), which include the GPI-anchored and transmembrane (TM) isoforms and the PCR products obtained with the region between the SCRV and ST-d regions (lanes 3 and 4), which include the ST-a, ST-ab, and ST-abc isoforms. A strong predominance of GPI-anchored isoforms was observed in normal gastric mucosa (lane 1), whereas a predominance of TM isoforms was observed in gastric mucosa subjected to I/R stress (lane 2). A predominance of the ST-ab isoform was observed in normal gastric mucosa (lane 3), whereas a predominance of the ST-abc isoform was observed in gastric mucosa subjected to I/R stress (lane 4).

Fig. 6B shows the PCR products obtained with the region between the SCR4 and G (19) regions and demonstrates the changes in expression of the 6 isoforms after I/R stress. TM-a and TM-ab isoforms were detected in normal mucosa, but TM-abc was not.

FIGURE 1. Changes in macroscopic damage after I/R. The damage after I/R was assessed from the area of macroscopic hemorrhages and erosions. The lesion index is the percentile of the lesion area to the total area of the stomach. Mucosal erosion was observed from 6 h to 3 days after I/R but was not detected at 6 days. Each value represents the mean ± SE of each group (\( n = 10 \)). * \( p < 0.05 \) vs day 0 and day 6 groups.

FIGURE 2. Mucosal epithelial injury after I/R. Histological changes were assessed using sections stained with hematoxylin and eosin. A, Mucosal erosion was not observed at 0 h. B, Mucosal erosion and neutrophil accumulation were observed at 6 h after I/R. C, Mucosal erosion was observed at 24 h after I/R. D, No erosion was observed at 6 days after I/R. (magnification, × 200).
However, by 6 h after I/R, mRNAs for TM-abc had appeared, and TM-a and TM-ab had become significantly dominant.

**Gastric mucosal distribution of DAF mRNA**

To determine the riboprobe specificity of the DAF mRNA to be used in ISH, Northern blot analysis was performed using the alkaline phosphatase-conjugated anti-digoxigenin Ab and chemiluminescent detection. Two bands were detected with the antisense riboprobe (Fig. 7), as in the case of Northern blot analysis using $^{32}$P-labeled cDNA probe (Fig. 5), whereas no bands were detected with the sense riboprobe (data not shown). Only a few signals specific for DAF mRNA were detectable in the normal gastric mucosal cells (Fig. 8A). A significant increase in specific signals was detected at 6 h after I/R. Strong expression was observed in the cytoplasm of the mucosal epithelial cells beneath the area of erosion 6 h after I/R (Fig. 8C). However, only a few signals specific for DAF mRNA were detectable 24 h after I/R (Fig. 8E). A negative control with the sense probe (Fig. 8, B, D, and F) and another without the anti-digoxigenin Ab (data not shown) showed no reactivity.

**FIGURE 3.** Immunohistochemical analysis of DAF. Immunohistochemical staining was performed with a specific mAb to GP DAF. A. Only a minute amount of DAF immunoreactivity can be observed in normal epithelial cells ($\times 200$). C. Strong DAF immunoreactivity is visible 24 h after I/R, predominantly on the luminal surface of epithelial cells (indicated by arrows). E. Only a minute amount of DAF immunoreactivity can be seen 6 days after I/R. Negative controls are to the right (B, D, and F).
Effects of complement depletion on I/R-induced gastric mucosal injury

When GPs were i.p. injected with 50 units of purified CVF, serum complement activity determined by the CH50 assay was found to have decreased to less than detectable levels within 30 min, and this condition lasted for at least 48 h (data not shown). When GPs were i.p. injected with 50 units of purified CVF 24 h before I/R, lesion indices were significantly reduced at 6, 12, or 24 h, or at 3 days after I/R (Fig. 9A). Expression of DAF in CVF-treated gastric epithelial cells was significantly reduced at 12 h or 24 h, or at 3 days after the I/R procedure (Fig. 9B).

FIGURE 7. Northern blot analysis of tissue expression of DAF mRNA. Blots of 20 μg of total RNA from each tissue were hybridized with digoxigenin-labeled antisense DNA of DAF, and DAF mRNA was detected by an enhanced chemiluminescence method as described in Materials and Methods (lane 1, liver; lane 2, stomach).
Discussion

Large amounts of DAF Ag have been detected on the surfaces of human epithelial cells in many extravascular sites throughout the body (3). Expression of DAF has also been analyzed in certain GP tissues (21). Recently, epithelial cells in various human tissues have been found to produce and secrete several complement components (7–9).

DAF, which restricts complement activation on homologous cell membranes, is present on the apical side of human gastric epithelial cells (22). Similarly, in epithelial cells of the colonic mucosa, DAF is localized to the apical surface (10, 23–25), and DAF expression has been found to be enhanced in colonic epithelial cells of patients with ulcerative colitis (24, 25). Furthermore, expression of DAF on gastric epithelial cells is strongly enhanced in gastritis (22, 26).

To study the possible changes in DAF expression in gastric mucosa of experimental animals under pathological conditions, we used a mAb against GP DAF (18).

GP DAF was weakly detected on epithelial cells of normal gastric mucosa by immunohistochemical staining with mAb. However, the extent of staining was significantly increased after I/R. Since the stress of undergoing I/R increases local blood circulation...
gastric mucosa, although the relative amounts of the various isoforms differed depending on the length of the period after I/R. The GPI-anchored form was found to be predominant in normal human intestinal tissue (28), and our study indicates that the GPI-anchored form is also clearly predominant in normal gastric mucosa of GPs. At 6 h after I/R, however, it was the TM form that predominated. Although there may be functional differences between GPI and TM forms of GP DAF, the length of the ST region significantly affected its inhibitory effect (28). In GPs, a longer ST region appears advantageous in protection against C-mediated cytolsis. The ST-abc form, which has the longest ST region, became relatively predominant after I/R (Fig. 6). GP polymorphonuclear cells and lymphocytes, cultured in RPMI 1640 (Nissui Pharmaceuticals, Tokyo, Japan) containing 10% FBS (Atlanta Biologicals, Atlanta, GA) for 6 h, expressed DAF largely in the TM form (data not shown). This suggests that, since the TM forms on leukocytes as well as on gastric mucosa are more sensitive to stimulation than GPI-anchored forms, TM isoform of DAF might play an important role as an acute phase reactant at the site of inflammation. The TM forms and longer ST forms may predominate when gastric injury occurs. However, the biologic implication of preferential expression of GPI and TM forms in gastric mucosa remains to be elucidated.

Localization of the cells that produce DAF mRNA was determined by ISH using the digoxigenin method (29). DAF expression was unclear in infiltrated neutrophils in the region surrounding the erosion. However, at 6 h after I/R, strong DAF expression was observed in the cytoplasm of cells beneath the eroded tissue (Fig. 8C). The evidence corresponded to the results of Northern blotting and RT-PCR.

A reduction in complement by administration of CVF inhibited shock syndrome induced by intestinal I/R (17). Therefore, we administered CVF before I/R to study the effect of complement in the regulation of GP DAF. The CVF dose used (50 U/GP, i.p.) reduced CH50 values to undetectable levels without any noticeable change in gastric mucosal histology (data not shown) or in the number of blood cells or platelets 12–24 h after injection (30). We failed to demonstrate a change in C3 expression in gastric mucosa with anti-GP C3 mAb due to the large amount of secreted C3 in the mucus, which resulted in high background staining. However, CVF administration resulted in a significantly smaller area of erosion after I/R (Fig. 9A), and up-regulation of DAF expression was limited. Therefore, up-regulation of gastric DAF may result from inflammatory tissue reactions although the factors that stimulate epithelial cells to up-regulate DAF expression remain to be identified.

**Acknowledgments**

We thank Dr. William Campbell and Catherine Campbell for English editing of this manuscript.

**References**


