International Immunopharmacology 15 (2013) 581–587

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Dextran sulfate-induced degradation of spontaneously apoptotic B cells

Yusuke Kadota, Nao Sakai, Ryoma Fujikawa, Eriko Aoyama, Ming Zhong, Satoshi Tanaka, Eiichi Gohda *

Division of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Tsushima-naka, Kita-ku, Okayama 700-8530, Japan

ARTICLE INFO

Article history:
Received 14 September 2012
Received in revised form 2 January 2013
Accepted 16 January 2013
Available online 31 January 2013

Keywords:
Dextran sulfate
B cell
Apoptosis
Degradation
Heparin

ABSTRACT

Mature resting mouse splenic B cells undergo spontaneous apoptosis in vitro, unless rescued by specific agents including interleukin 4 and protein kinase C activators. Dextran sulfate, a B cell activator, has been reported to have such a protective effect on B cell apoptosis. This study was undertaken to elucidate the mechanism underlying the protective effect of dextran sulfate. The ratio of apoptotic B cells gradually increased to about one third with 24 h of incubation. Dextran sulfate dose-dependently reduced apoptotic cells, but it did not cause concomitant increase in viable cells. Both DNA levels and lactate dehydrogenase activities in the supernatants of dextran sulfate-treated cultures were significantly higher than those in the supernatants of untreated cultures. Concomitantly, DNA levels and lactate dehydrogenase activities in the cell pellets of dextran sulfate-treated cultures were lower than those in the cell pellets of untreated cultures. Addition of dextran sulfate to the culture of B cells 18 h after the start of incubation, when about one fifth of the B cells were dead, significantly reduced apoptotic cells during the next 6-h incubation. This decrease in the number of apoptotic cells was detectable as early as 1 h after addition of dextran sulfate and was prevented by Zn^{2+}, Co^{2+}, Ni^{2+}, the serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) or incubation on ice. These results indicated that dextran sulfate treatment did not prevent apoptosis but rather promoted degradation of apoptotic cells and suggest the involvement of DNase and protease in this process.

1. Introduction

The proliferation and survival of a B cell population must be well regulated to avoid undesired responses. Regulation of apoptosis in the B cell lineage has implications for homeostasis, quality control of the antibody response, and tolerance [1]. Several lines of evidence suggest that Fas participates in regulating the elimination of activated, tolerant and autoreactive B cells [2–4]. Mature, recirculating B cells exhibit low levels of Fas expression [5]. However, B cell stimulation as a result of CD40 engagement by CD40 ligand is accompanied by induction of Fas expression and susceptibility to Fas-mediated apoptosis [6,7]. Thus, CD40/CD40 ligand interaction would promote B cell growth and differentiation, followed perhaps by increased Fas expression to limit clonal expansion. B cells sensitized to Fas-mediated apoptosis can be protected by signals from the B cell receptor or the type 2 T helper cell (Th2) cytokine interleukin 4 (IL-4) [8,9]. In addition to Fas signaling, it has been suggested that ligation of B cell receptor (BCR) with antigens inactivates or eliminates mature B cells [10–12]. Antigen-stimulated B cells, however, may escape elimination by interacting with T helper cells through CD40 or IL-4 receptor engagement [13].

In vitro mature resting mouse splenic B cells undergo spontaneous apoptosis, unless rescued by specific agents including IL-4 and protein kinase C activators [14]. The number of spontaneous apoptotic B cells in vitro increases gradually and monotonically [14]. Dextran sulfate has been reported to decrease apoptotic cells, indicating that it has such a protective effect against spontaneous apoptosis of B cells, although its mechanism is unknown [15,16]. Dextran sulfate is a T cell-independent type 2 antigen, which has a highly repetitive structure. Its multiple and identical epitopes are believed to bring about efficient cross-linking of BCR, thereby delivering prolonged and persistent signaling to the B cells and stimulating proliferation of B cells and antibody production [17–19]. This study was undertaken to elucidate the mechanism underlying the protective effect of dextran sulfate. Surprisingly, our results demonstrated that dextran sulfate did not inhibit apoptosis but rather promoted degradation of apoptotic B cells, resulting in reduction of apoptotic B cells.

2. Materials and methods

2.1. Reagents

Dextran sulfate sodium salt (MW 5×10^5, 10^4 and 2.5×10^3), dextran (MW 5×10^5), heparin sodium salt (porcine intestinal mucosa), lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, annexin V-fluorescein isothiocyanate (FITC), propidium iodide (PI), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), aprotinin, bestatin, and pepstatin A were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Leupeptin and E-64 were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Hamster anti-mouse CD40, anti-mouse CD16/CD32

* Corresponding author. Tel.: +81 86 238 9425; fax: +81 86 251 7926.
E-mail address: gouda-e@cc.okayama-u.ac.jp (E. Gohda).

1567-5769/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.intimp.2013.01.013

and FITC-conjugated rat anti-mouse CD45R/B220 monoclonal antibodies (mAbs) were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

2.2. Mice

Female BALB/c mice, purchased from Charles River Japan (Yokohama, Japan), were maintained under specific pathogen-free conditions in the animal facility of Okayama University and used between 8 and 12 weeks of age. Experimental procedures involving mice were approved by the Animal Research Control Committee of Okayama University.

2.3. Preparation of B cells

Murine spleen resting B cells were purified by negative selection using a mouse B lymphocyte enrichment set (BD Biosciences) as described previously [20]. Briefly, murine spleen cells depleted of erythrocytes by lysis with ammonium chloride were suspended in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and incubated on ice for 60 min with biotin-conjugated anti-mouse CD4, CD43 and TER-119/erythroid cell mAbs. The cells bearing the biotinylated antibodies were bound to BD IMag streptavidin particles, and negative selection was then performed on a BD IMagnet according to the manufacturer's protocol to enrich unlabeled B cells. The purity of recovered viable B cells was more than 95% when the cells were stained with FITC-conjugated anti-mouse CD45R/B220 mAb and PI after preincubation of the cells with anti-mouse CD16/CD32 mAb to block Fc-mediated binding of Ab to the Fcγ receptor of cells and analyzed by a flow cytometer (Epics XL, Beckman Coulter, Miami, FL, USA). More than 95% cells in the enriched B cell preparations were small, resting B cells as judged by the cell size (forward scatter) in flow cytometry.

2.4. Culture of B cells

B cells were suspended in basal culture medium (RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/mL penicillin G and 100 μg/mL streptomycin) and incubated for various periods in 24-well plates (2×10^6 cells/1 mL/well) at 37 °C in an atmosphere containing 5% CO_2. In some cultures, Phenol Red-free RPMI 1640 medium was used instead of RPMI 1640 medium.

2.5. Flow cytometric analysis of apoptotic B cells

Apoptotic cells were determined as described previously [21]. Briefly, B cells incubated with reagents for various periods in 24-well plates were centrifuged, washed twice with phosphate-buffered saline (PBS), and fixed with 70% ethanol overnight at −30 °C. After being washed with PBS, the cells were incubated with ribonuclease A (0.1 mg/mL) in 200 μL of PBS for 30 min at 37 °C. The cells were then washed with and suspended in 0.5 mL of PBS and further incubated with PI (50 μg/mL) for 30 min at room temperature in the dark. Cells were filtered through a nylon mesh, and the DNA contents of cells were analyzed by an Epics XL flow cytometer. Cells with a sub-G1 DNA content were regarded as apoptotic cells.

Apoptotic cells were also determined by the annexin V binding assay [22]. Briefly, B cells incubated with reagents in the basal culture medium for 8 h in 24-well plates were centrifuged and washed twice with PBS. The cells were suspended in 10 mM HEPES buffer (pH 7.5) containing 140 mM NaCl and 2.5 mM $CaCl_2$ and incubated with annexin V-FITC (50 ng/mL) for 10 min on ice. The cells were then labeled with PI (2 μg/mL) and analyzed by an Epics XL flow cytometer. Annexin V-positive/PI-negative cells were regarded as early apoptotic cells.

2.6. Determination of DNA

B cell cultures incubated with or without dextran sulfate in the Phenol Red-free basal culture medium for 24 h in 24-well plates were centrifuged. The supernatants diluted with an equal volume of 1 N perchloric acid and the cell pellets suspended in 0.5 N perchloric acid were incubated at 70 °C for 20 min to hydrolyze DNA. Then the mixture was cooled and centrifuged, and the supernatant was used for measurement of DNA. DNA was assayed by the modified diphenylamine reaction described by Giles and Myers with calf thymus DNA as a standard [23].

2.7. Determination of lactate dehydrogenase

B cell cultures incubated with or without dextran sulfate in the Phenol Red-free basal culture medium for 16 h in 24-well plates were centrifuged. Lactate dehydrogenase activity in the supernatant and the cell pellets was measured by the Cytotoxicity Detection Kit^{plus} (LDH) (Roche Diagnostics Japan, Tokyo, Japan) according to the manufacturer's instructions.

2.8. Determination of DNA synthesis

B cells, suspended in the basal culture medium, were cultured with or without dextran sulfate for 45 h in triplicate in flat-bottom 96-well plates (4×10^5/0.2 mL/well). The cells were then pulse-labeled with [^{3}H]thymidine (0.5 μCi/well, 2.5 Ci/mmol) for 3 h and harvested on glass fiber filters as described previously [20]. The amount of [^{3}H]thymidine incorporated was measured in a liquid scintillation counter.

2.9. Data analysis

All results are expressed as means and SD of three independent experiments except the results shown in Fig. 3A, which are expressed as those of four independent experiments. Data in two groups were analyzed by Student's *t*-test. Multiple comparison of the data was done by ANOVA followed by Tukey's test or Dunnett's T3-test. *p* values less than 5% were regarded as significant.

3. Results

3.1. Decrease in apoptotic B cells in dextran sulfate-treated cultures without concomitant increase in viable cells

Murine splenic B cells in culture are known to undergo spontaneous apoptosis that proceeds gradually and monotonically [14]. B cells were incubated for 24 h with or without dextran sulfate, and apoptotic cells with a sub-G1 DNA content were determined by flow cytometric analysis. About one third of the B cells in control cultures had died after incubation for 24 h, but addition of dextran sulfate at the start of incubation reduced apoptotic cells in a dose-dependent manner as reported by Mower et al. (Fig. 1A) [15]. Apoptotic cells were reduced to 23% and 8% at 5 and 10 μg/mL of dextran sulfate, respectively, and minimal apoptotic cells were detected at 30 and 100 μg/mL. Early apoptotic cells with annexin V-positive/PI-negative labeling were also decreased by the treatment with dextran sulfate (Fig. 1B and C). We used dextran sulfate with an MW of 5×10^5 in this study, the effect of which was much greater than the effects of dextran sulfate with a lower MW (10^4 or 2.5×10^3) (Fig. 1D). In contrast, nonsulfated dextran with an average MW of 5×10^5 was ineffective (data not shown). Heparin, a sulfated glycosaminoglycan, was also effective but less potent than dextran sulfate (Fig. 1A). Dextran sulfate is known as a B cell activator, which stimulates proliferation of B cells [17]. This stimulatory effect of dextran sulfate, however, was minimal at 10 μg/mL but