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Calcium-dependent proapoptotic effect of *Taenia solium* metacestodes annexin B1 on human eosinophils: A novel strategy to prevent host immune response

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ABSTRACT

Annexins are a family of calcium-dependent phospholipid-binding proteins that have been proposed to be involved in a wide range of important biological processes. At present, only a few annexins have been identified in parasites, and the physiological roles of these annexins are obscure. Earlier, we cloned a novel annexin (annexin B1) from *Taenia solium* metacestodes and found that annexin B1 was detectable in the surrounding host-derived layer with granulomaous infiltration. The objective of this study was to investigate the secretion and physiological function of annexin B1. We expressed a green fluorescent protein-tagged annexin B1 (GFP-anxB1) in living SiHa cells and showed that it was secreted upon stimulation with dexamethasone (Dex). This secretion was not inhibited by brefeldin A but was blocked by pre-treatment with the intracellular calcium-specific chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA/AM). Furthermore, we describe for the first time that annexin B1 can bind to the extracellular surface of human eosinophils and produce Ca^{2+}-influx. The Ca^{2+}-influx induced apoptosis in eosinophils, which was inhibited by pre-loading the Ca^{2+} channel blocker 1-[β-[3-(4-methoxyphenyl)propoxy]-4-metho-xyphenethyl]-1*H*-imidazole, HCl (SKF-96365). In conclusion, these findings represent direct and substantial evidence for the secretion of annexin B1 by living cells; the apoptosis in eosinophil induced by annexin B1 might be a novel strategy for *T. solium* metacestodes to prevent the host's immune attack.

1. Introduction

Annexins, which are expressed throughout the animal and plant kingdoms, are a family of proteins capable of binding to negatively charged phospholipids in a Ca^{2+}-dependent manner. Annexins have been proposed to have a wide range of important biological roles, such as membrane trafficking and fusion, Ca^{2+} channel activity, inhibition of protein kinase C, anti-inflammatory activity, interaction with cytoskeletal proteins, etc. (Dubois, Oudinet, Mira, & Russo, 1996; Gerke & Moss, 2002; Hayes & Moss, 2004; Raynal & Pollard, 1994). Although these proteins lack secretory signals, many reports have documented that some annexins, such as A1, A5, A6, and A2, are present

Abbreviations: anxB1, annexin B1; BAPTA/AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester; Bref A, brefeldin A; DMEM, Dulbecco's modified Eagle's medium; Dex, dexmethasone-21-phosphate; FACS, flow cytometric analysis; FBS, fetal bovine serum; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; SKF, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-metho-xyphenethyl]-1*H*-imidazole, HCl.

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in the extracellular milieu (Hajjar, Jacovina, & Chacko, 1994; Rocha, Lozano, & Haindl, 1990; Vergnolle, Pages, & Guimbaud, 2004). Since the lack of a hydrophobic leader peptide prevents them from being targeted to the endoplasmic reticulum, annexins are not secreted through the classical exocytic pathway, and an unknown alternative pathway is generally admitted (Chander, Sen, & Spitzer, 2001; Christmas, Callaway, Fallon, Jones, & Haigler, 1991).

Earlier, we cloned the novel annexin B1 from a *Taenia solium* cysticercus cDNA library (Genbank accession no. AF147955) (Yan, Sun, Chen, & Guo, 2002). Annexin B1 is expressed stage-specifically in *T. solium* metacestodes and accounts for 2–3% of total cysticercus proteins (Wang, Peng, Yao, & Sun, 1996), implying that it might have a vital role in the life-cycle of *T. solium* metacestodes (Winter, Yusof, Gao, Yan, Sun, & Hofmann, 2006). Intriguingly, annexin B1 has been detected in the cystic fluid of cysticerci, and in the sera of pigs and humans with cysticercosis; immunohistochemical analysis revealed that annexin B1 was present in the surrounding host-derived layer with granulomatous infiltration. These findings indicated that annexin B1 might be secreted from the cells (Gao, Yan, Ding, Lu, & Sun, 2007). However, immunocytochemical analysis is limited to fixed tissues; therefore, besides the secretion of annexin B1, specific immuno-reactivity in the inflammatory host cells might be due to these phagocytosing parasite cells. More studies should be done to determine whether annexin B1 is secreted by living cells.

At present, only a few annexins have been identified in parasites (Creutz, Snyder, Daigle, & Redick, 1996; Weilandm, Palmm, Griffithsm, McCafferym, & Svardm, 2003), and the physiological roles of these annexins are unknown. Earlier, we found that the secreted annexin B1 was co-localized with eosinophils in the host–parasite interface with high granulomatous infiltration. Eosinophils have a critical role in the immune response to infection with helminths (Wardlawm, Moqbelm, & Kaym, 1995). The ability of these cells to damage or kill the larval stages of helminth parasites has been demonstrated by *in vitro* assays, mainly by using an antibody-dependent cellular cytotoxity mechanism (Rainbird, Macmillan, & Meeusen, 1998). On the other hand, helminth infections are chronic disease, which implies the development of strategies by the parasites that avoid or prevent immune attack by the host. Therefore, the observation that secreted annexin B1 is co-located with eosinophils suggests that annexin B1 might have a role in the parasite's immune evasion. The objective of this study was to determine whether annexin B1 is secreted from living cells, and to investigate the possible role of the secreted annexin B1 in the parasite's evasion of the immune response of the host.

2. Materials and methods

2.1. Materials

Calcium ionophore A23187, dexamethasone (Dex) (water soluble), 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA/AM), 1-[β-[3-(4-methoxyphenyl)propoxy]-4-metho-xyphenethyl]-1*H*-imidazole, HCl (SKF-96365), brefeldin A and Hoechst H33342 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 were from GIBCO™ Invitrogen (Carlsbad, CA, USA). Mouse monoclonal antibody against annexin B1 (MoAb, 2B10H5) was prepared earlier (Gao et al., 2007). Recombinant annexin B1 (purity >98%) was produced in *Escherichia coli* and purified by FPLC as described (Zhang, Guo, Sun, Yan, & He, 2004). Endotoxin contamination was <20 pg/ml, as measured by the *Limulus* amebocyte chromogenic assay.

Dex and SKF-96365 were dissolved in water, calcium ionophore A23187, brefeldin A and BAPTA/AM were dissolved in dimethylsulfoxide (DMSO) to give stock solutions and then added to the culture medium to give a range of indicated concentrations (DMSO final concentration <0.1%). In each experiment, controls were always included by incubating the cells in the corresponding volume of culture medium plus the appropriate vehicle. The effect of pharmacological treatments on cell viability was monitored by the trypan blue exclusion and thiazolyl blue tetrazolium bromide (MTT; Sigma–Aldrich, St. Louis, MO, USA) reduction test before each experiment.

2.2. SiHa cells culture, transfection and confocal imaging

To construct eukaryotic expression plasmids, the full-length cDNA sequence of annexin B1 was amplified by PCR and subcloned into the *Eco*RI/*Sal*I sites of pEGFP-N1 (Clontech, USA) and transformed into *E. coli*. DH5α. The recombinant pEGFP-anxB1 was confirmed by DNA sequencing.

SiHa cells were cultured to 80% confluence with DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C. For transient transfections, the recombinant plasmid pEGFP-anxB1, or the empty vector pEGFP-N1 was transfected into SiHa cells using the Lipofectamine™ and PLUS™ reagents (Invitrogen, USA) according to the protocol provided by the manufacturer. After transfection for 24 h, 10^5 cells were seeded onto thin glass coverslips in 12-well plates and cultured overnight. In the next day, the coverslips were washed twice in PBS and then the cells were incubated in Hepes buffer (135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Hepes, 10 mM glucose, pH 7.2) and exposed to different stimuli for the indicated lengths of time at 37 °C. Then the cells were fixed with 3.7% paraformaldehyde. Fluorescent images were collected using a confocal laser scanning microscope (TCS SP2 from Leica Microsystem; 488 nm argon excitation, 515 nm long pass barrier filters).

2.3. Immunogold staining and electron microscopy

pEGFP-anxB1 transfected cells were incubated in the presence or in the absence of 0.8 μM Dex for 8 h, then fixed using 1.5% paraformaldehyde, 1% glutaraldehyde, 0.13% picric acid in 0.1 M phosphate buffer (pH 7.4), and dehydrated through an ascending series of ethanol concentrations (5–100%). Immunogold staining and electron microscopy were done as described (Faure, Migne, Devilliers, & Ayala, 2002), with the primary antibody against annexin B1 (MoAb, 2B10H5, 1:500 diluted in PBS)

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and protein A-gold conjugate (10 nm diameter grains from Beyotime Institute of Biotechnology, Beijing, China). The sections were viewed with an electron microscope (Philips 410 LS) operated at 60–80 kV. The pEGFP-N1-transfected SiHa cells were used as a negative control.

2.4. Western blot analysis

pEGFP-anxB1 transfected cells were treated with the indicated stimuli, then the cells and the medium were separated and collected by centrifugation at 400 × *g* for 5 min. The harvested cells (6×10^6) were washed twice with Hepes buffer (containing 4 mM EGTA) and the washings were collected. The medium and the washings were filtered gently through a 0.2 μm pore size membrane to eliminate any detached cells. The supernatants were concentrated in the presence of 4 mM EGTA and Protease Inhibitor Cocktail. The corresponding cells were lyzed in 10 mM Tris–HCl (pH 7.6), 10% glycerol, 5 mM β-mercaptoethanol and Protease Inhibitor Cocktail. Samples of the cellular lysates (25 μg) and EGTA elutes were subjected to SDS-10% PAGE, transferred to PVDF membrane (Amersham Pharmacia Biotech) and incubated with a mouse anti-anxB1 monoclonal antibody (2B10H5, 1:500 dilution) for 4 h; followed by incubation with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000 dilution, Sigma, USA). The immune complexes were visualized by enhanced chemiluminescence-based detection (Amersham Pharmacia Biotech).

2.5. Isolation of peripheral blood eosinophils

The research protocol and consent forms were approved by the Ethics Review Committee of the Second Military Medical University. With informed consent, eosinophils were isolated from samples of peripheral blood of healthy individuals, using immunomagnetic beads and the magnetic cell separation system (Eosinophil isolation kit, Miltenyi Biotech, Bergisch Gladbach, Germany) according to the protocol provided by the manufacturer. Briefly, anticoagulated blood was diluted 1/1 in PBS with 2 mM EDTA and layered onto Percoll (density 1.087 g/ml; pH 7.4; Amersham Pharmacia Biotech). Following centrifugation at 600 × *g* for 30 min, carefully remove and discard the plasma, the mononuclear cells and Ficoll-Paque, leaving the red cell pellet undisturbed. Resuspend the red cell pellet in crythrocyte lysis solution (0.8% NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and incubate for 10 min on ice. Then the cells were centrifuged at 300 × *g* for 8 min, and the cell pellet was suspended and incubated with Biotin-Antibody Cocktail (10 μl/10^7 cells) for 10 min at 4 °C, then Anti-Biotin MicroBeads were added (20 μl/10^7 cells). The cells were resuspended and loaded onto the MACS column. The column was eluted with PBS buffer (pH 7.2, containing 0.5% BSA and 2 mM EDTA) and the enriched eosinophil cells passed through the column. The concentration of cells was adjusted to 5×10^6/ml in RPMI-1640 supplemented with 10% FBS, 2 mM gluthamine and 50 μg/ml gentamycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. The purity and viability of the eosinophils were >95%, as confirmed by Wright's stain and trypan blue dye exclusion, respectively. Purified eosinophils were used immediately for following experiments.

2.6. Annexin B1 binding assay

The annexin B1 binding assay was done essentially as described (Euzger, Flower, Goulding, & Perretti, 1999; Le, Mishal, Lemonnier, & Kourilsky, 1983) but with some modifications. Eosinophils were resuspended at 5×10^6 cells/ml in RPMI-1640 containing 0.2% BSA and 2 mM $CaCl_2$ or 2 mM EGTA. Aliquots of 20 μl were added to 96-well plates and incubated with 20 μl of purified recombinant annexin B1 (concentrations range from 0 to 1 μM) at 37 °C for 30 min. Cells were then washed and 20 μl of human IgG (10 mg/ml in PBS containing 0.2% BSA and 2 mM $CaCl_2$ or 2 mM EGTA.) was added to the same buffer to block non-specific sites. Then 20 μl of a specific mouse monoclonal antibody against annexin B1 (2B10H5) (50 μg/ml) was added and the mixture was incubated at 37 °C for 30 min. Finally, the cells were washed three times with PBS and incubated with 20 μl of an anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (1:30 dilution in PBS) (Sirocco Biotechnology Co., Shanghai, China) for 30 min. The cells were fixed in 1% paraformaldehyde and analyzed by flow-cytometry within 12 h (FACSCalibur, BD Bioscience, San Jose, USA).

2.7. Apoptosis detection

2.7.1. Hoechst nuclear staining

Cells were counterstained with Hoechst 33342 to detect condensation and chromatin fragmentation of the nuclei. Control or treated cells were re-suspended in 50 μl RPMI-1640 medium at a concentration of 1×10^6 cells/ml and 5 μl of a solution of Hoechst H33342 was added (final concentration 1 μg/ml). The cells were incubated for 20 min at 37 °C and then fixed with 10 μl of cold formaldehyde (40%, w/v). Control cells and treated cells (at least 1000 of each) were examined with a Leica fluorescence microscope.

2.7.2. PI/FITC-ANXA5 binding assay

To differentiate between early and late apoptosis, we quantified the externalized phosphatidylserine (PS) by FITC-annexin A5 (Annexin A5-Fluos, 1/50, Boehringer Mannheim) according to the manufacturer's protocol. In brief, control or treated eosinophils (1×10^6 cells/ml) were washed in cold PBS and resuspended in 100 μl binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) in the presence of FITC-annexin A5 and propidium iodide (PI), and incubated at room temperature for 15 min in darkness. Analysis by flow-cytometry was done within 1 h. FITC-annexin A5 staining was detected in the FL1 channel, and PI staining was monitored in the FL2 channel. The following controls were used to set up compensation and quadrants: unstained cells and cells stained with FITC-annexin A5 or with PI alone. Other samples were incubated with FITC-annexin A5 in buffer without calcium to control for non-specific binding.

2.7.3. Caspase 3 activity assay

Eosinophils were preloaded with or without 25 μM SKF for 30 min followed by incubation with 0.6 μM annexin

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B1 for the indicated time (0, 6, 12, 18, 24 h) at 37 °C. The cells were collected by centrifugation at 200 × *g* for 10 min at 4 °C, washed twice with ice-cold PBS (pH 7.4), followed by centrifugation at 200 × *g* for 5 min. Caspase 3-like activity was measured as described (Enari, Hug, & Nagata, 1995). Briefly, cells were lyzed in extraction buffer (50 mM Pipes-NaOH (pH 7.0), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT and Protease Inhibitor Cocktail). Cell extracts (25 μg of protein) were incubated at 30 °C for 30 min with 2 mM MCA-DEVDAPK(dnp)-NH_2, a specific fluorescent substrate for caspase 3, in assay buffer (100 mM Hepes-KOH (pH 7.5), 10% sucrose, 0.1% Chaps, 10 mM DTT, 0.1 mg/ml of ovalbumin). The fluorescence of the cleaved substrate was measured at an excitation wavelength of 325 nm and an emission wavelength of 392 nm. One unit of enzyme was defined as the amount of enzyme that cleaves 1 pmol of the fluorescent substrate at 30 °C in 30 min under standard reaction conditions.

2.7.4. Cytochrome c detection

For the detection of cytochrome *c*, cells (5×10^6) treated with 0.6 μM annexin B1, 0.6 μM annexin B1+ 25 μM SKF or control were collected by centrifugation at 200 × *g* at 4 °C. Cells were resuspended in ice-cold cell extraction buffer (20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 100 mM PMSF and Protease Inhibitor Cocktail) for 30 min on ice. Cells were then homogenized with a glass Dounce homogenizer and centrifuged at 1000 × *g* to remove unbroken cells, pellet nuclei, and heavy membranes. The post nuclear supernatant was centrifuged at 14,000 × *g* for 30 min to yield a pellet of the mitochondria-enriched heavy membrane fraction, and the resulting supernatant was centrifuged at 100,000 × *g* to obtain the cytosolic fraction. Supernatants were then frozen in aliquots at −70 °C. The mitochondria-rich fraction was washed once with extraction buffer, followed by a final resuspension in lysis buffer (150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 1 mM EGTA) containing protease inhibitors. Samples of cytosolic or mitochondrial protein extracts (~5 μg) were separated by 15% SDS-PAGE and transferred to nitrocellulose. The blots were incubated with monoclonal anti-cytochrome *c*, anti-caspase 3, anti-Cox-4, and β-actin followed by enhanced chemiluminescence-based detection (Amersham Pharmacia Biotech).

2.8. Measurement of intracellular calcium in cells

To measure the changes of $[Ca^{2+}]_i$, the intracellular concentration of Ca^{2+}, in response to different stimuli, cells were preloaded with 3 μM fura-2/AM at 37 °C, then washed rapidly by centrifugation and resuspended in Hepes buffer. After stimulation, the $[Ca^{2+}]_i$ measurements were done with 2 ml of cell suspension (1×10^6 cells) in a luminescence spectrometer (Perkin Elmer LS 50) at a constant temperature of 37 °C. The excitation wavelength was 340 nm and the emission wavelength was 510 nm. The $[Ca^{2+}]_i$ corresponding to fluorescence emitted by trapped fura-2 was calculated by the equation:

$$[Ca^{2+}]_i = 224\,\text{nM} \times \left(\frac{F - F_{\min}}{F_{\max} - F}\right)$$

$$F_{\min} = \text{AF} + \left[\frac{F_{\max} - \text{AF}}{3}\right]$$

where 224 nM is the K_d value for fura-2, $F_{\max}$ and AF (autofluorescence) were obtained after the addition of ionomycin (3 μM) and $MnCl_2$ (2.25 mM), respectively, as described (Duarte, Ferreira, Santos, Oliveira, & Carvalho, 1993).

2.9. Statistical analysis

Data are expressed as mean ± S.E.M of *n* experiments performed in triplicate. Statistical comparisons were made with Student's *t*-test (two treatment groups) or one-way analysis of variance (ANOVA). Any difference for which $P \leq 0.05$ was regarded as statistically significant.

3. Results

3.1. Externalization of GFP-anxB1 in SiHa cells upon stimulation with Dex

To observe directly whether annexin B1 was secreted from living cells, we generated a fusion protein of green fluorescent protein with annexin B1 (GFP-anxB1). Western blot analysis revealed that GFP-anxB1 was detected by antibodies against GFP and against annexin B1, indicating that GFP-anxB1 was expressed as an intact protein (data not shown).

SiHa cells transfected with pEGFP-anxB1 or pEGFP (control) were incubated in the absence or in the presence of different concentrations of Dex (0.2–1.0 μM). As shown in Fig. 1A, large numbers of fluorescence particles were present on the outer membrane of the cells after incubation with 0.8 μM Dex for 8 h. By contrast, the distribution of fluorescence intensity in pEGFP-N1 transfected SiHa cells was unchanged after the same treatment. Although 0.4 μM Dex appeared to externalize the GFP-anxB1 from SiHa cells, a significant and measurable effect was observed for 0.8 μM Dex. Therefore, 0.8 μM Dex was chosen as the concentration to be used for the following studies.

To determine whether GFP-anxB1 was present on the outer membrane, we used immunogold electron microcopy to study Dex-stimulated cells. As shown in Fig. 1B, in cells treated with 0.8 μM Dex for 8 h, numerous immunogold particles were present both in the cytoplasm and at the external sides of the membranes; immunogold particles were observed mainly in the cytoplasm of non-stimulated cells. Immunogold particles were not detected in cells transfected with pEGFP-N1, indicating that the fluorescent particles are specific signals of GFP-anxB1.

The externally bound GFP-anxB1 can be removed by washing the cells with a Ca^{2+}-free salt solution (containing 4 mM EGTA), which chelates Ca^{2+} and releases

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the extracellular GFP-anxB1 into the medium. The corresponding cellular lysates were used for the detection of intracellular GFP-anxB1. Western blot analysis (Fig. 1C) showed that GFP-anxB1 was detected after treatment with 0.8 μM Dex for 30 min, and the amount of proteins released increased with incubation time (4 h and 8 h) ($P < 0.01$ vs. control).

The GFP-anxB1 released is unlikely to be caused by leakage of the proteins from dead or damaged cells, as the viability of the cells treated with Dex for 30 min, 4 h, and 8 h was always >95%, as determined by trypan blue exclusion and by the MTT assay.

3.2. Effect of the $[Ca^{2+}]_i$ on secretion of GFP-anxB1

Annexins are Ca^{2+}-dependent phospholipid-binding proteins and most of the annexin-associated physiological processes are dependent on $[Ca^{2+}]_i$; therefore, we assessed

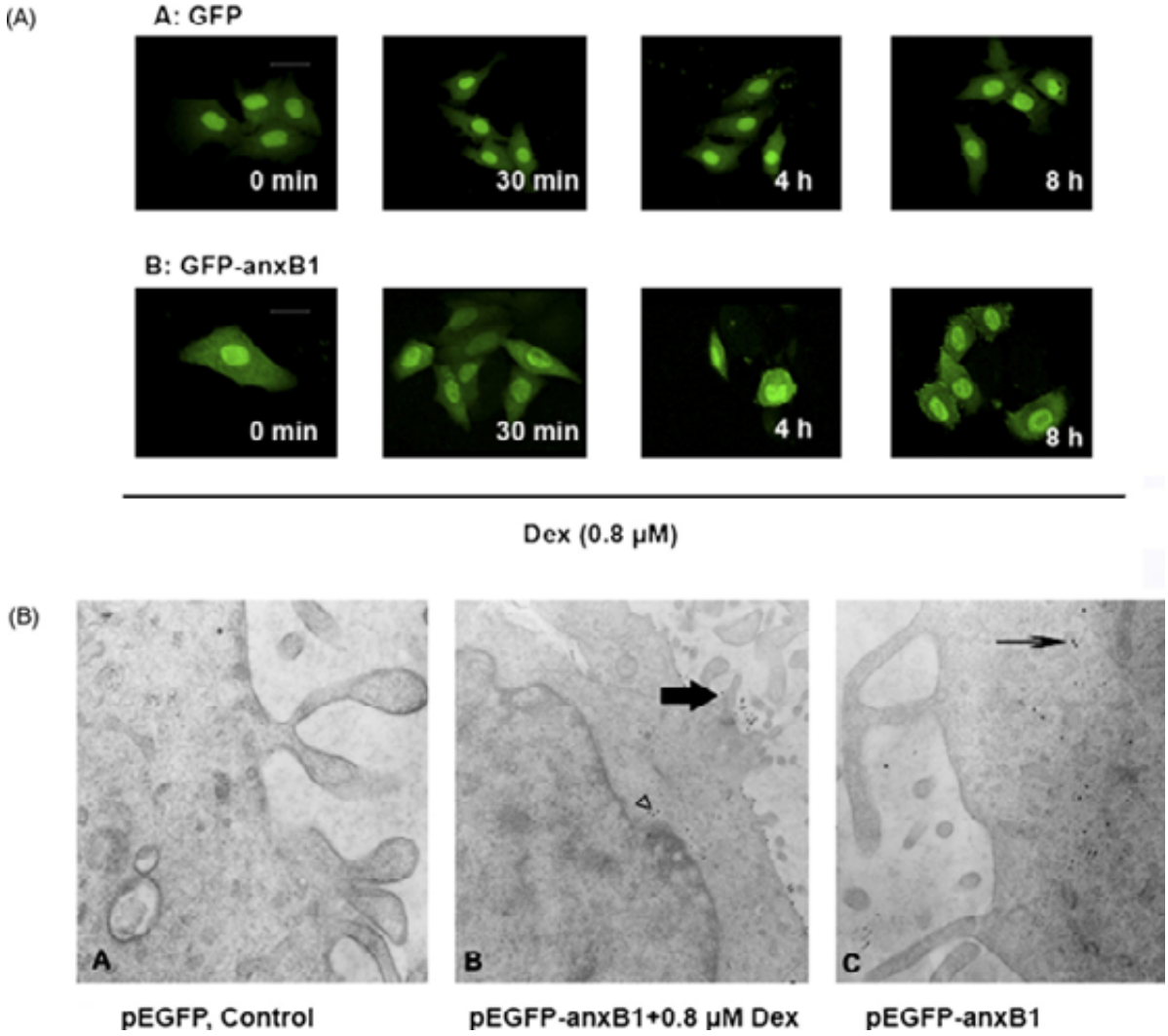

Fig. 1. Dex induced GFP-anxB1 externalization in SiHa cells. (A) Confocal fluorescence microscopy. pEGFP-anxB1 or pEGFP transfected SiHa cells were treated with 0.8 μM Dex for 30 min, 4 h and 8 h. The treated cells were fixed with 3.7% formaldehyde and the fluorescence in the cytosol of the membrane was examined by confocal laser scanning microscopy. The scale bars represent 20 μm. (B) Immunogold electron microscopy. pEGFP-anxB1 or pEGFP transfected SiHa cells were treated with 0.8 μM Dex for 8 h, then the treated cells were fixed. The procedure of immunogold staining and detection is described in Section 2. (A) pEGFP transfected cells, control. (B) pEGFP-anxB1 transfected cells treated with Dex. The thick arrow and the framed triangle show that the gold grains were present on the outside of the plasma membrane and in the cytoplasm. (C) pEGFP-anxB1-transfected cells without treatment with Dex. The thin arrow shows that the gold grains were present mainly in the cytoplasm. Magnification of photos: (A) 15,000×; (B) 10,000×; (C) 15,000×. (C) Western blot analysis. pEGFP-anxB1 transfected cells were incubated in the absence (control, C) or in the presence of 0.8 μM Dex. After treatment for 30 min, 4 h and 8 h, GFP-anxB1 bound externally to plasma membranes was removed by washing with a solution of EGTA, as described in Section 2. The EGTA eluates and cellular lysates (25 μg) were determined by Western blot analysis, using a mouse monoclonal anti-annexin B1 antibody, and the EGTA elutes bands were quantified with an image analyzer. The data shown are an average of three independent experiments. $^{**}P < 0.01$ vs. control. (D) Effect of Dex on the $[Ca^{2+}]_i$. pEGFP-anxB1 transfected SiHa cells were incubated in the absence (control, C) or in the presence of 0.8 μM Dex for 30 min, 4 h and 8 h. The resulting $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator fura-2 and calculated as described in Section 2. The data shown are an average of three independent experiments. $^{**}P < 0.01$ vs. control.

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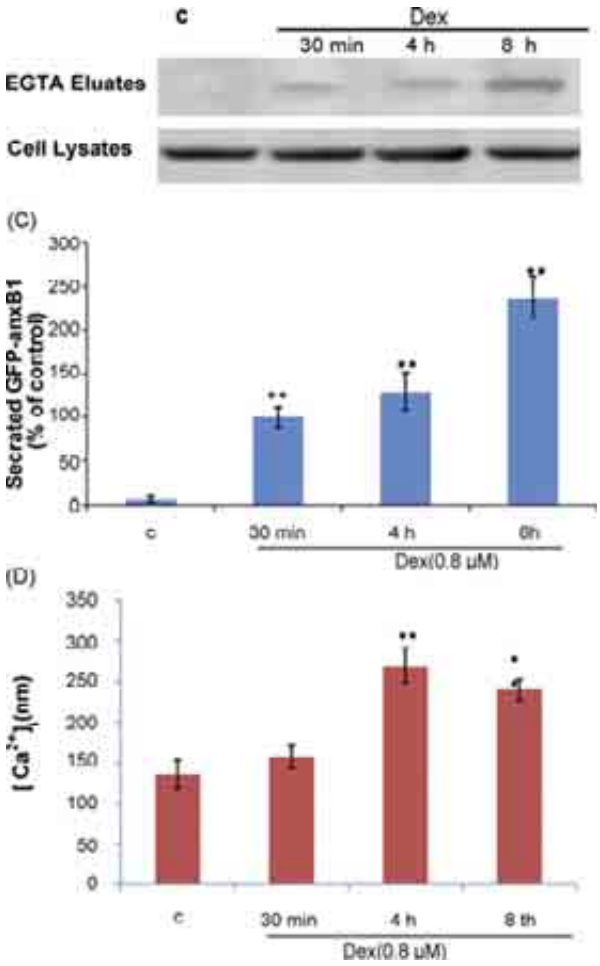

Fig. 1. (*Continued*).

$[Ca^{2+}]_i$ by using the fluorescent indicator fura-2/AM. As shown in Fig. 1D, $[Ca^{2+}]_i$ was increased significantly after treatment with 0.8 μM Dex for 4 h and for 8 h ($P < 0.01$ vs. control).

To further study whether the effect of Dex on the secretion of GFP-anxB1 could be attributed to the increase of $[Ca^{2+}]_i$, pEGFP-anxB1 transfected cells were pre-loaded with 10 μM Ca^{2+} chelator BAPTA/AM before stimulation by Dex. As shown in Fig. 2A (top panel) and B, no translocation or secretion of GFP-anxB1 was observed. In addition, when the cells were stimulated with Ca^{2+} ionophore A23187, which can increase the $[Ca^{2+}]_i$ rapidly, although GFP-anxB1 was induced to translocate from the cytoplasm to the plasma or nuclear membrane, no externalization of GFP-anxB1 was observed (Fig. 2A (bottom panel) and Fig. 2C). Therefore, an increase of $[Ca^{2+}]_i$ is necessary but not sufficient for the secretion of GFP-anxB1.

To elucidate the mechanism by which Dex stimulates the secretion of GFP-anxB1, we used brefeldin A (Bref A) to block the classical endoplasmic reticulum–Golgi pathway of protein secretion. As shown in Fig. 3, Bref A at concentrations ranging from 0.1 to 1 mM was unable to inhibit the Dex-induced secretion of GFP-anxB1 significantly ($P > 0.05$ vs. control).

3.3. Annexin B1 bound to eosinophils and produced a Ca^{2+} influxes

Our earlier study showed that annexin B1 was co-localized with neutrophils and eosinophils at the host–parasite inflammatory sites. We incubated annexin B1 with human eosinophils to study the possible physiological functions of the secreted annexin B1. As shown in Fig. 4, annexin B1 bound to the eosinophils in a concentration-dependent manner (0–1 μM). The binding ability was abolished in the presence of 4 mM EGTA, indicating that the binding of annexin B1 to eosinophils is Ca^{2+}-dependent.

To investigate the response of eosinophils after binding annexin B1, we measured the changes of the cytosolic free calcium. As shown in Fig. 5, binding annexin B1 resulted in a transient increase in $[Ca^{2+}]_i$ in a concentration-dependent manner ($P < 0.05$ vs. control). Pre-loading with the calcium-entry blocker SKF-96365 inhibited annexin B1-induced Ca^{2+} influx.

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3.4. Annexin B1 binding induced apoptosis of eosinophils

Because changes in $[Ca^{2+}]_i$ have been linked to induction of apoptosis (Mattson & Chan, 2003), we investigated whether human eosinophils incubated with annexin B1 would accelerate apoptosis in eosinophils. As shown in Fig. 6A, incubation with 0.6 μM annexin B1 resulted in a time-dependent acceleration of apoptosis in eosinophils.

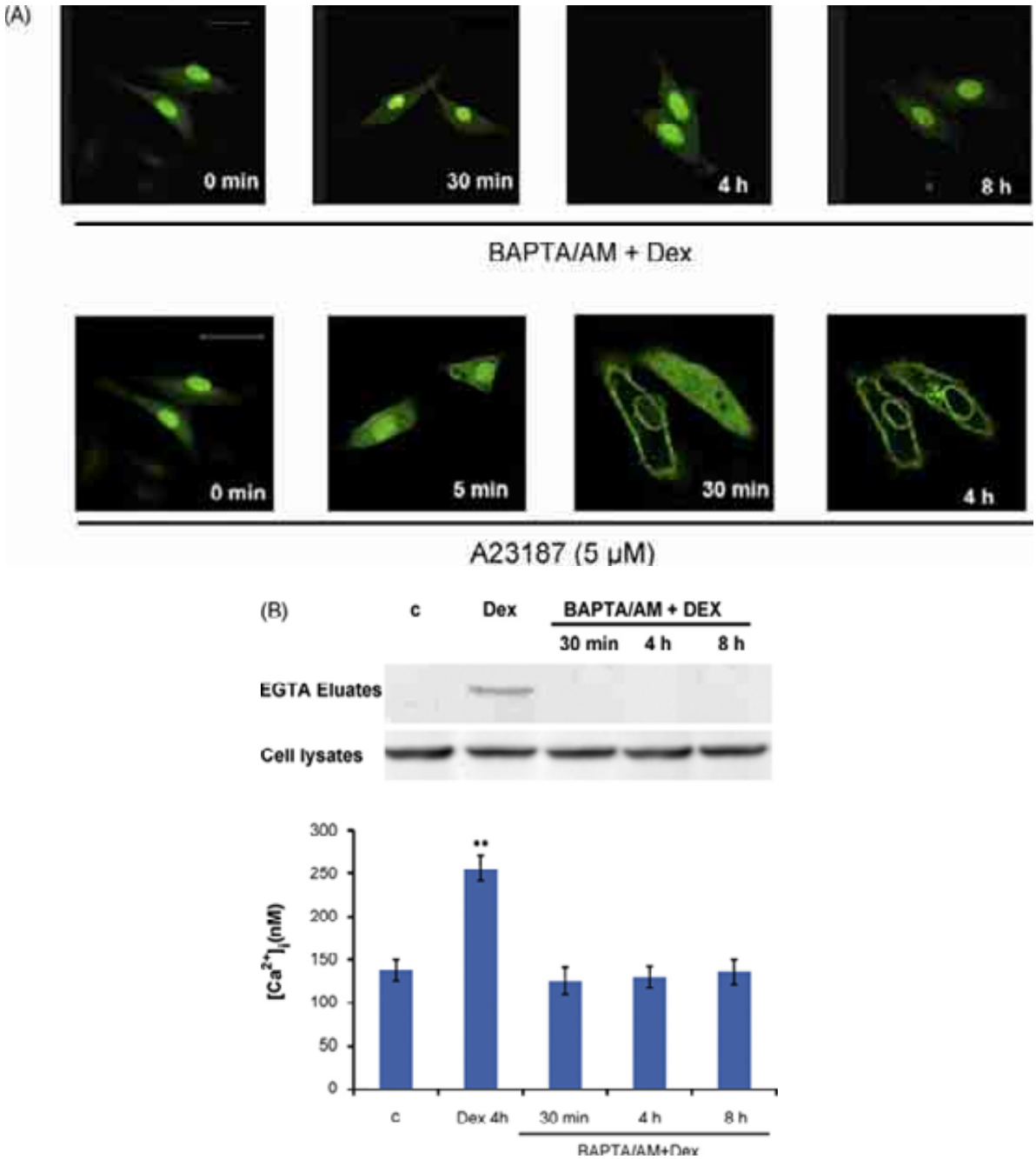

Fig. 2. Effect of Ca^{2+} ionophore A23187 and Ca^{2+} chelator BAPTA/AM on the externalization of GFP-anxB1 in SiHa cells. pEGFP-anxB1 transfected SiHa cells were co-stimulated with 10 μM BAPTA/AM and 0.8 μM Dex for 30 min, 4 h and 8 h, or treated with 5 μM A23187 for 5 min, 30 min and 4 h. (A) Confocal fluorescence microscopy. The treated cells were fixed with 3.7% formaldehyde and the fluorescence in the cytosol of the membrane was examined by confocal laser scanning microscopy. The scale bars represent 20 μm. Top panel: treated with BAPTA/AM + Dex; bottom panel: treated with A23187. The secretion of GFP-anxB1 and the resulting $[Ca^{2+}]_i$ after treated with BAPTA/AM + Dex (B) or A23187 (C) was examined. GFP-anxB1 bound externally to the plasma membranes was removed by washing with a solution of EGTA, as described in Section 2. The EGTA eluates and cellular lysates (25 μg) were determined by Western blot analysis, using a mouse monoclonal anti-annexin B1 antibody. The resulting $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator fura-2. The data shown are an average of three independent experiments. $^{**}P < 0.01$ vs. control.

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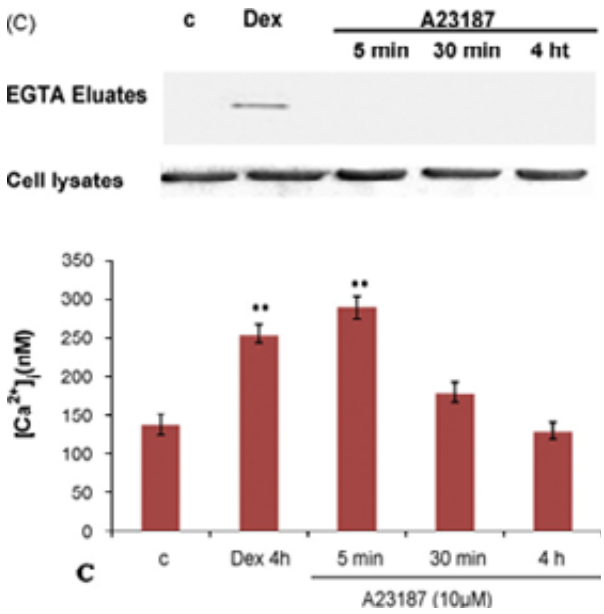

Fig. 2. (*Continued*).

When eosinophils were incubated with 0.6 μM annexin B1 for 3 h, apoptosis was induced in 54% of eosinophils, a significantly higher value of 2.5% for the control ($P < 0.001$). After incubation with annexin B1 for 24 h, there was apoptosis in 90% of eosinophils but in only 22% of cells in the control. Therefore, we chose 3 h as the length of incubation in the following studies. As shown in Fig. 6B, incubation with different concentrations of annexin B1 for 3 h resulted in a concentration-dependent acceleration of apoptosis in eosinophils. Therefore, the apoptosis of eosinophils induced by annexin B1 was time and concentration-dependent, which was susceptible to inhibition by SKF-96365. Fig. 6C shows representative pictures

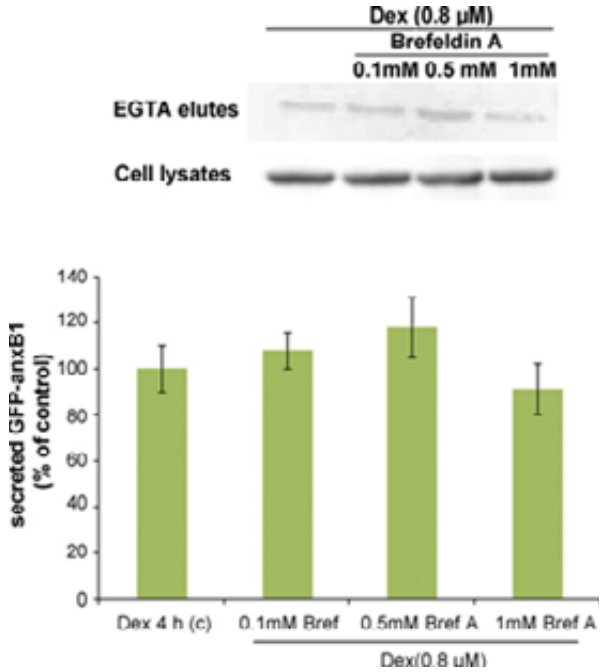

Fig. 3. Effect of brefeldin A on the secretion of GFP-anxB1 in SiHa cells. pEGFP-anxB1 transfected cells were incubated without (control) or with 0.8 μM Dex in the absence or in the presence of brefeldin A (0.1–1 mM) for 4 h. GFP-anxB1 bound externally to plasma membranes was removed by washing with a solution of EGTA, as described in Section 2. The EGTA eluates and cellular lysates (25 μg) were determined by Western blot analysis, using a mouse monoclonal anti-annexin B1 antibody. The EGTA eluate bands were quantified with an image analyzer. The data shown are an average of three independent experiments. $^{**}P < 0.01$ vs. control.

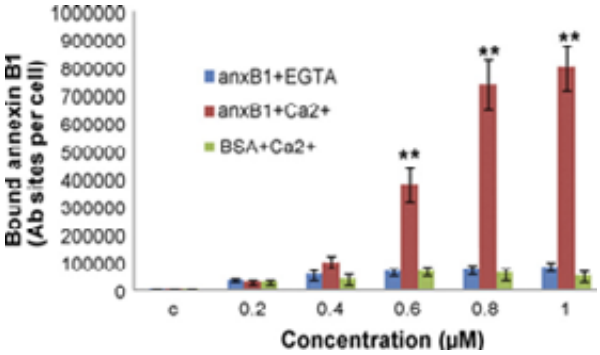

Fig. 4. Annexin B1 bound to eosinophils in a concentration-dependent manner. Human eosinophils were isolated as described in Section 2 and incubated with different concentrations of recombinant annexin B1. BSA was used as a negative control. Incubation was in culture media containing 2 mM $CaCl_2$ (anxB1 + Ca^{2+}) or 4 mM EGTA (Ca^{2+}-free buffer, anxB1 + EGTA). Binding was detected by indirect immunofluorescence plus quantitative flow-cytometry and is expressed as secondary antibody molecules bound per cell. The values are mean ± S.E.M., $n = 10$ subjects. $^{**}P < 0.01$ vs. BSA control.

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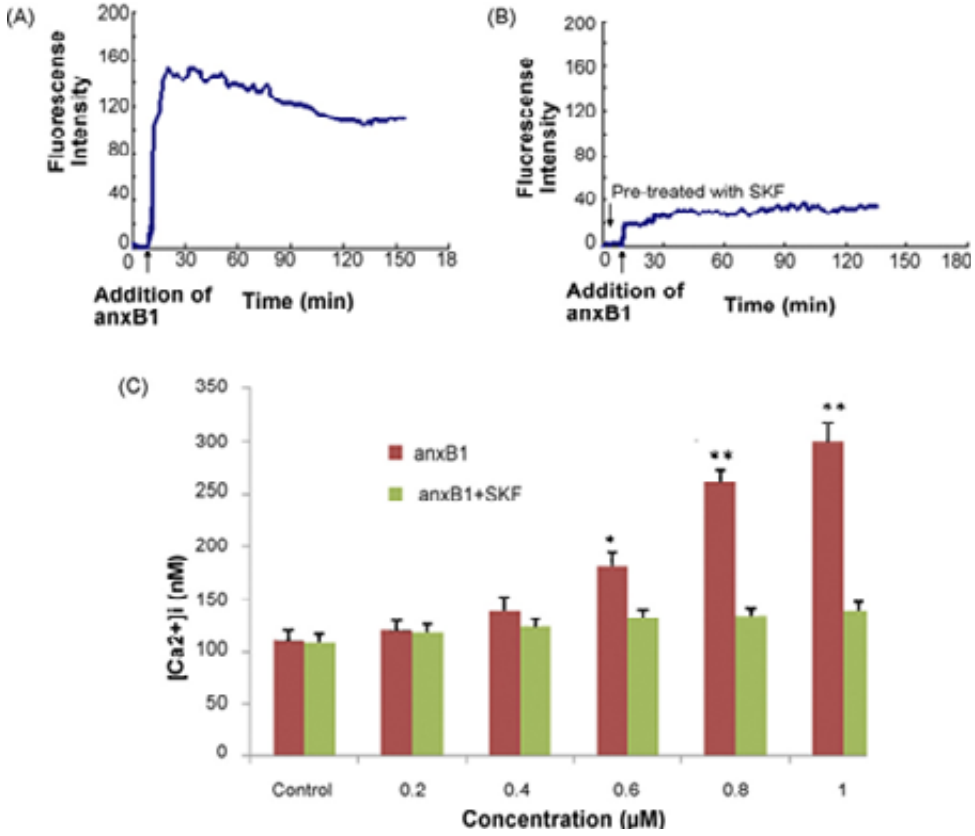

Fig. 5. Annexin B1 binding to human eosinophils activates transient Ca^{2+} influxes. Eosinophils were loaded with fura-2 and incubated with different concentrations of annexin B1, and the changes of $[Ca^{2+}]_i$ were recorded. (A) Representative of the $[Ca^{2+}]_i$-dependent fluorescence changes in a single donor treated with 0.6 μM annexin B1; (B) pre-loading with 25 μM Ca^{2+} blocker SKF-96365 before stimulation with 0.6 μM annexin B1. (C) Concentration-related response for annexin B1 pre-loading with or without 25 μM Ca^{2+} blocker SKF-96365. The data shown are averages of six independent experiments. The values are mean ± S.E.M. $^{**}P < 0.01$ vs. control.

of nuclear fragmentation as visualized by Hoechst staining, and Fig. 6D and E shows representative double staining with FITC-ANXA5 and PI.

Caspase 3 activity after incubation with annexin B1 was significantly higher than that measured in control cells (Fig. 7A). The amount of cytochrome *c* was increased after annexin B1 binding to eosinophils and was decreased after blocking Ca^{2+} influx with SKF-96365 (Fig. 7B). These data suggested that apoptosis in eosinophils might be associated with Ca^{2+} influx and a mitochondrial signaling pathway.

4. Discussion

To observe the secretion of annexin B1 in living cells directly, we used a green fluorescent protein-tagged annexin B1 (GFP-anxB1) to study its cellular distribution and secretion. The fusion protein and GFP alone were expressed transiently in SiHa cells, and their intracellular localization was monitored by the bright green fluorescence emitted from the GFP tag. We used Dex as a stimulus on the basis of the following facts: we showed earlier that annexin B1 was able to inhibit phospholipase A2 activity and might have a vital role in anti-inflammatory action (Huang, Wang, Sun, & Yan, 2005). Some annexins (e.g. annexin A1) have been shown to be important mediators in the anti-inflammatory action of glucocorticosteroids (Alldridge, Harris, Plevin, Hannon, & Bryant, 1999; Goulding, Euzger, Butt, & Perretti, 1998). Dex, a synthetic glucocorticosteroid, increased $[Ca^{2+}]_i$ and induced expression and secretion of annexin A1 (Castro, Duarte, Carvalho, & Lopes, 2002; Solito, Raugei, Melli, & Parente, 1991); therefore, we determined whether GFP-anxB1 was released from the SiHa cells upon stimulation by Dex.

The results were very interesting: GFP-anxB1 was externalized to the outer surface of the plasma membrane when stimulated with Dex, which was identified by confocal imaging, immunoelectron microscopy and by Western blot analysis (Fig. 1). The secretion of GFP-anxB1 was blocked completely when the cells were pre-loaded with the selective Ca^{2+} chelator BAPTA/AM. However, when $[Ca^{2+}]_i$ was increased by the Ca^{2+} ionophore A23187, no secretion of GFP-anxB1 was observed. Therefore, increased $[Ca^{2+}]_i$ is necessary but not sufficient for the secretion of GFP-anxB1, which implies that, besides this Ca^{2+}-dependent signaling pathway, other intracellular processes have been activated by Dex that allow secretion of annexin B1. Although this pathway needs further investigation, these findings, in addition to the results of our earlier studies, provide evidence that annexin B1 was indeed secreted to the extracellular milieu.

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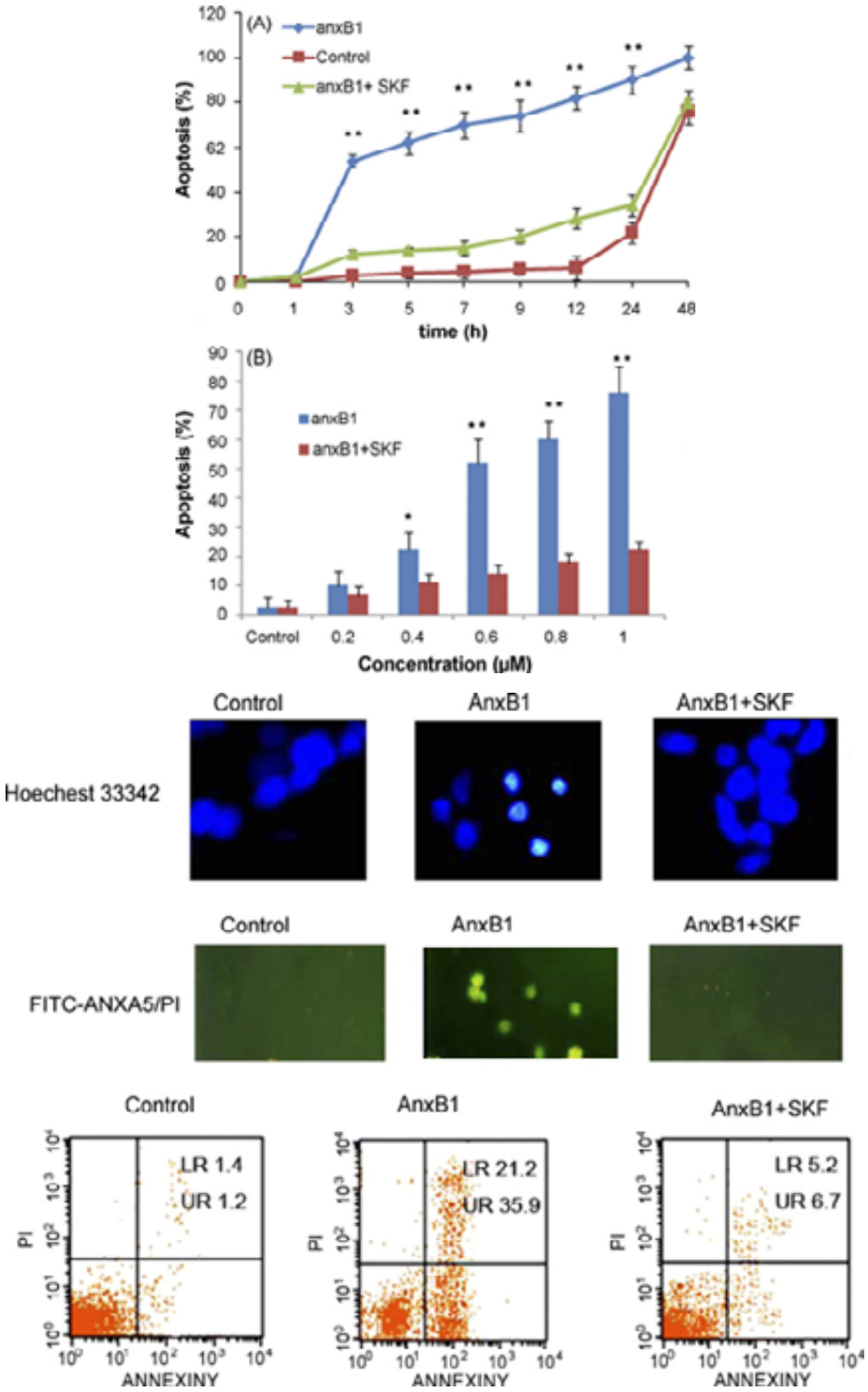

Fig. 6. Annexin B1 induced apoptosis in human eosinophil. (A) Apoptosis in eosinophils induced by annexin B1 is time-dependent. Eosinophils were cultured in medium alone (control), or in the presence of 0.6 μM anxB1 or 0.6 μM anxB1 + 25 μM SKF for different lengths of time (0–48 h). (B) Apoptosis in eosinophils induced by annexin B1 is concentration-dependent. Eosinophils were cultured in medium alone (control), or in the presence of increasing concentrations of anxB1 (0.2–1 μM) pretreated with or without 25 μM SKF for 3 h at 37 °C. The degree of cell apoptosis was measured by annexin A5/PI staining and analyzed by flow-cytometry. The values are mean ± S.E.M., $n = 6$ subjects. $^{**}P < 0.01$ vs. control. (C) Representative Hoechst immunostaining of control, treatment with 0.6 μM annexin B1 or treatment with 0.6 μM annexin B1 + 25 μM S KF. (D) Representative annexin A5/PI staining of previous donor. (E) Dot plots showing annexin A5 (FL1 channel) and PI (FL2 channel) staining.

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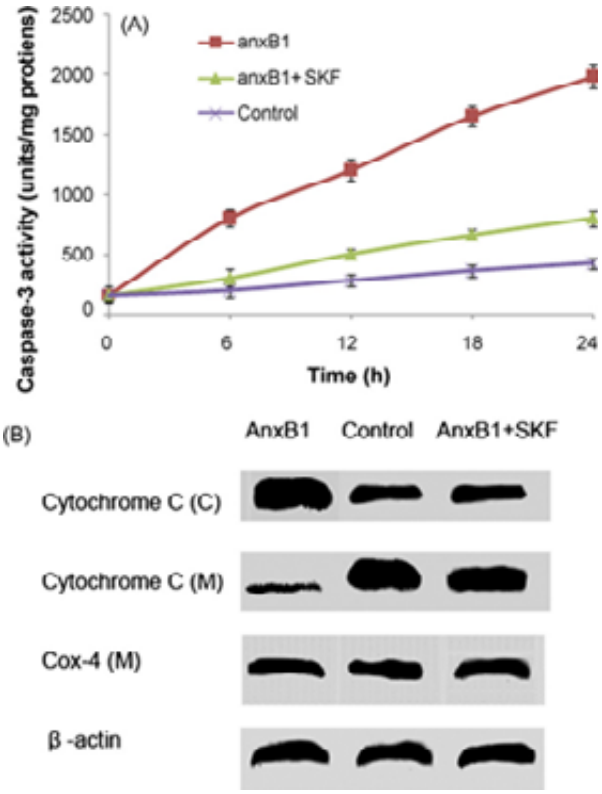

Fig. 7. Inductions of caspase 3 activity and cytochrome *c* release during apoptosis of eosinophils. Eosinophils were preloaded with or without 25 μM SKF for 30 min, then incubated with 0.6 μM annexin B1 for 3 h. Cells treated with medium alone were used as a control. The caspase 3 activity assay and Western blot detection of cytochrome *c* are described in Section 2. (A) Caspase 3 activity was measured with a fluorescent substrate specific for caspase 3. The results shown are typical of results obtained in three independent experiments. $^{*}P < 0.05$ vs. control. (B) Representative Western blot experiments of cytochrome *c* release in the cytosol. Mitochondial (M) and cytosolic (C) fractions were prepared as described in Section 2. Equal amounts of protein from mitochondrial or cytosolic fractions were separated by SDS-15% PAGE, transferred to nitrocellulose membranes, and immunoblotted with the indicated cytochrome *c* antibody. Cox-4 and β-actin were used as internal controls.

The mechanism of annexins secretion is still an enigma. Christmas et al. (Christmas et al., 1991) reported that the human prostate gland selectively secretes high concentrations of annexin A1. Blockage of the classical endoplasmic reticulum–Golgi pathway of protein secretion does not affect the secretion of annexin A1 (Philip, Flower, & Buckingham, 1998). Deora et al. (Deora, Kreitzer, Jacovina, & Hajjar, 2004) indicated that endothelial cell annexin A2 is translocated from the cytoplasm to the extracytoplasmic plasma membrane in response to a brief temperature stress, and this response does not require the classical endoplasmic reticulum–Golgi pathway. In contrast, Wang et al. (Wang, Campos, Kaetzel, & Dedman, 2001) designed transgenes of annexin V with or without an attached secretory signal peptide and investigated the secretion of the transgene products in COS-7 cells. They found that secretion of annexin V from cultured cells requires a signal peptide. In the present study, GFP-anxB1 has no signal peptide, but it could be secreted upon stimulation by Dex. When Dex-treated cells were incubated with Bref A, an agent that disrupts the endoplasmic reticulum–Golgi complex, no decrease in the secretion of GFP-anxB1 was observed, suggesting that annexin B1, just like other annexins (e.g. annexins A1 and A2), has an non-classic secretion pathway. Because annexin B1 is an annexin from a parasite, and there is no endogenous expression of annexin B1 in human SiHa cells, the SiHa cells transfected with pEGFP-anxB1 are a good model for investigating the mechanisms of annexin secretion.

The next question to be resolved is why annexin B1 was secreted mainly in early cysticercosis and was present at the site of inflammation It is well known that *T. solium* metacestodes, like many other helminth parasites, are long-lived and can survive in the host without causing a severe manifestation of disease, which implies that the parasites have developed some strategy to avoid or prevent an immune attack from the host. It has been demonstrated recently that parasite-induced apoptosis of host cells is one of the mechanisms of immunosuppression (Chin, Teoh, Scott, Meddings, Macnaughton, & Buret, 2002; James & Green, 2004; Pinheiro, Pinto, Benedito, Lopes, & Rossi, 2004; Wei et al., 2002). However, little is known about the induction of host cell apoptosis by helminthic worms.

Here, we showed that annexin B1 was able to bind to the extracellular surface of eosinophils and produce a Ca^{2+} influx. Thymocytes and eosinophils have been shown to enter into apoptosis following a rise in $[Ca^{2+}]_i$ (Kubista, Hawkins, Patel, Haigler, & Moss, 1999; Ward, Dransfield, Chilvers, Haslett, & Rossi, 1999). Intriguingly, a Ca^{2+}-influx after annexin B1 binding was also able to induce apoptosis in eosinophils. The apoptosis was inhibited by SKF, a Ca^{2+}-channel blocker. Eosinophils possess at least two different modes for apoptosis: caspase 3-mediated apoptosis (Min et al., 2004) and caspase 3 independent apoptosis (Zhang, Wong, & Lam, 2000). We found increased caspase 3 activity and cytochrome *c* release in annexin B1-induced apoptosis of eosinophil, indicating that this apoptosis is caspase 3-dependent and associated with a mitochondrial signaling pathway. These findings are in line with other reports, in which a participation of caspase 3 was also observed in the eosinophil apoptosis induced by excretory-secretory products of newly excysted metacercariae of *P. westermani* (Min et al., 2004).

We found also that annexin B1 has a proapoptotic effect on human peripheral blood neutrophils, but not lymphocytes (unpublished results). On the basis of these findings, we propose a scheme for the possible function of the secreted annexin B1 (Fig. 8). In the early stage of *T. solium* metacestodes infection, the cystic annexin B1 externalized to the luminal side of tegument upon stimulation of the host inflammatory mechanism. The secreted annexin B1 binds to the eosinophils at the site of inflammation and induces apoptosis in eosinophils and neutrophils, and then the apoptotic cells are removed by the resident phagocytes. In this way, secreted annexin B1 down-regulates the host immune response and benefits survival of *T. solium* metacestodes. Further studies will clarify the details of this pathway.

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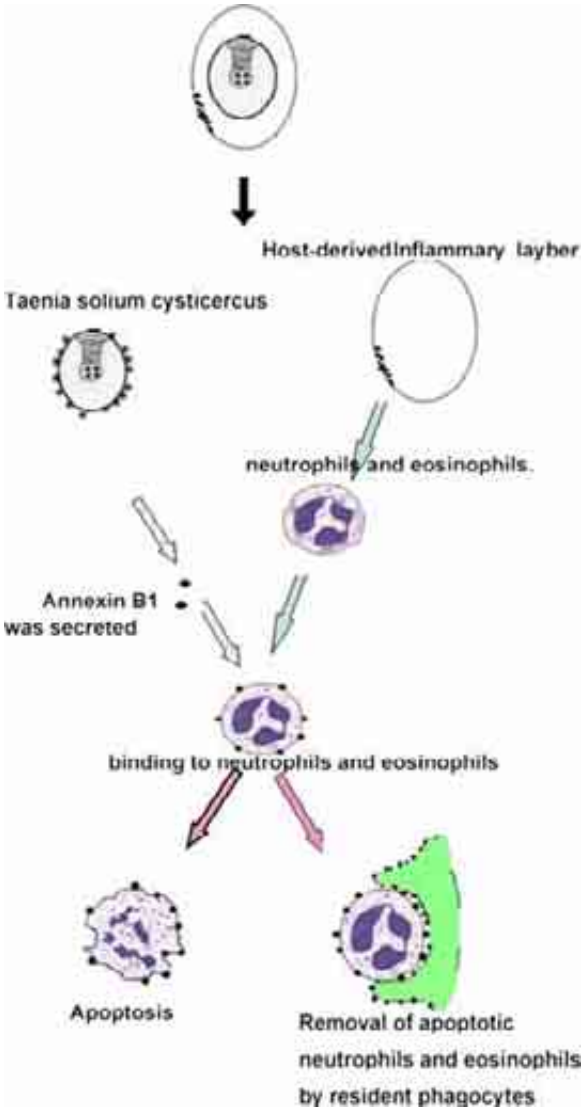

Fig. 8. Hypothesis for annexin B1 down-regulating host immune response. In early cysticercosis, annexin B1 was secreted upon host inflammatory stimulation, and then the secreted annexin B1 could bind to the eosinophils and neutrophils and induce apoptosis in these cells. Thus annexin B1 might down-regulate the host immune response by inducing apoptosis in eosinophils and neutrophils in the inflammatory site around *Taenia solium* metacestodes.

In conclusion, we showed that GFP-anxB1 was secreted upon stimulation by Dex, which provided direct and substantial evidence for the secretion of annexin B1 in living cells, and provided a good model for studying the mechanism of annexin secretion. Furthermore, we have described for the first time that annexin B1 can bind to the extracellular surface of human eosinophils and produce a Ca^{2+} influx, and then induce apoptosis of eosinophils. Eosinophils are well known to be the important immunoeffector cells in the host defense against helminth parasites. Therefore, apoptosis of eosinophils induced by the secreted annexin B1 might be a novel strategy for *T. solium* metacestodes to prevent immune attack by the host.

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