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Anti-SSA/Ro and Anti-SSB/La Autoantibodies Bind the Surface of Apoptotic Fetal Cardiocytes and Promote Secretion of TNF-α by Macrophages

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Despite the near universal association of congenital heart block and maternal Abs to SSA/Ro and SSB/La, the intracellular location of these Ags has made it difficult to substantiate their involvement in pathogenicity. To define whether components of the SSA/Ro-SSB/La complex, which translocate during apoptosis, are indeed accessible to extracellular Abs, two approaches were taken: immunoprecipitation of surface biotinylated proteins and scanning electron microscopy. Human fetal cardiocytes from 16–24-wk abortuses were cultured and incubated with staurosporine to induce apoptosis. Surface biotinylated 48-kDa SSB/La was reproducibly immunoprecipitated from apoptotic, but not nonapoptotic cardiocytes. Surface expression of SSA/Ro and SSB/La was further substantiated by scanning electron microscopy. Gold particles (following incubation with gold-labeled sera containing various specificities of anti-SSA/Ro-SSB/La Abs and murine mAb to SSB/La and 60-kDa SSA/Ro) were consistently observed on early and late apoptotic cardiocytes. No particles were seen after incubation with control antisera. To evaluate whether opsonized apoptotic cardiocytes promote inflammation, cells were cocultured with macrophages. Compared with nonapoptotic cardiocytes or apoptotic cardiocytes incubated with normal sera, apoptotic cardiocytes preincubated with affinity-purified Abs to SSB/La, 52-kDa SSA/Ro, or 60-kDa SSA/Ro increased the secretion of TNF-α from cocultured macrophages. In summary, apoptosis results in surface accessibility of all SSA/Ro-SSB/La Ags for recognition by circulating maternal Abs. It is speculated that in vivo such opsonized apoptotic cardiocytes promote an inflammatory response by resident macrophages with damage to surrounding conducting tissue. The Journal of Immunology, 2000, 165: 5345–5351.

Neonatal lupus is considered a model of passively acquired autoimmunity whereby maternal IgG Abs reactive with components of the SSA/Ro-SSB/La ribonucleoprotein complex are transported across the placenta via Fcγ receptors on the trophoblast into the fetal circulation (1). This active placental transport is effective by 11 wk of gestation (2). Fetal and neonatal disease appears to be totally independent of maternal health status since the mothers can have systemic lupus erythematosus, Sjögren’s syndrome, or in fact be clinically asymptomatic (3–5). The two classic manifestations of neonatal lupus are cardiac and cutaneous (1, 6), the former more serious and, with rare exception, permanent. Cardiac injury characteristically involves the conduction system and presents as congenital heart block (CHB),3 which can be first degree, but is most often second or third degree (7). CHB is not associated with major structural heart defects and is typically identified between 18 and 24 wk of gestation (8). Despite the nearly universal association of CHB with very specific maternal autoantibody responses, molecular mechanisms are only recently being explored to explain how the targeted Ags, 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro, which are normally sequestered within the confines of the nucleus and cytoplasm, become accessible to this immune response (9, 10).

The traditional paradigm to account for tissue damage caused by the binding of autoantibodies to intracellular Ags is that immune complexes form in the intravascular space with subsequent deposition of the complexes, activation of complement, and influx of inflammatory cells (11). Alternatively, Abs could directly bind to the cell surface and/or penetrate across the membrane. It has recently been reported that anti-P Abs penetrate living cells and inhibit protein synthesis (12). In consideration of surface binding, one hypothesis is that apoptosis, a physiologic process of remodeling during embryogenesis (13, 14), might result in translocation of intracellular Ags to the external leaflet of the membrane. Rosen et al. (9) first demonstrated, by confocal microscopy, the presence of SSA/Ro and SSB/La in surface blebs of apoptotic keratinocytes. This observation was extended to the developing fetal heart in which we documented, by indirect immunofluorescence, that anti-SSA/Ro and anti-SSB/La Abs recognize the surface of nonpermeabilized cultured human fetal cardiocytes (10). Relevant to CHB, apoptosis occurs in scattered cells rather than tracts of contiguous cells in a tissue (15). Therefore, binding of autoantibodies to the surface of apoptotic cells could trigger an inflammatory response that results in damage to surrounding healthy tissue. It logically follows that organs with minimal regenerative capacities such as the heart, and particularly the specialized cells of the conduction system, would be most vulnerable.
The present study was initiated to determine whether Abs reactive to each or all of the components of the SSA/Ro-SSB/La system truly bind to the surface of human fetal cardiocytes following the induction of apoptosis. Given the availability of a wide variety of human and murine polyclonal Abs and mAbs, two initial approaches were exploited. The methods included biotinylation of cell surface proteins and scanning electron microscopy of immunogold-labeled cells. Subsequent experiments addressed the consequences of surface binding by examining the release of the inflammatory cytokine, TNF-α, from macrophages cocultured with apoptotic fetal cardiocytes.

Methods and Isolations
Human fetal cardiocytes were cultured as described (16) with minor modifications. Briefly, human fetal hearts aged 16–24 wk were aseptically obtained after elective termination of normal pregnancy by dilatation and evacuation. This was done in accordance with the guidelines of the Institutional Review Board and after obtaining consent from the mothers. No cardiac toxic drugs were administered to the mothers during these procedures. The aorta was cannulated for continuous perfusion of the coronary arteries with calcium-free Tyrode’s solution (117 mM NaCl, 5.7 mM KCl, 1.1 mM glucose, 4.4 mM NaHCO3, 1.5 mM K2HPO4, 1.7 mM MgCl2, 20 mM NaH2PO4, pH 7.4) containing 1 U/ml of sodium-heparin at 37°C, bubbled with 100% O2, as described for the Langendorff preparation (17).

After 15 min of washing to clear the blood from the heart, fresh calcium-free Tyrode’s solution containing 1.5 mg/ml collagenase A (type III) was recirculated for approximately 20 min. The heart dissociated spontaneously, allowing cells to slowly drip and fall on a petri dish containing 0.25% trypsin, 1 mM EDTA in HBSS. Clumps of cells were dissociated and the resulting suspension poured over a cell strainer. Cells were centrifuged and the pellet resuspended in 20 ml of culture medium (DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 U/ml streptomycin, 100 mg/ml gentamicin, 1 mM nonessential amino acid (Life Technologies, Gaithersburg, MD), 0.1 mM essential medium vitamins (Life Technologies), 2 mM glutamine, and 0.1 mM sodium pyruvate). Cells were then plated at approximately 1.2 × 105 cells per 75-cm2 culture flask and grown in 5% CO2 at 37°C. After 4 days in culture, spontaneous contraction (30–40 beats per min) could be observed under phase-contrast microscopy.

Induction of apoptosis
Apoptosis was induced as previously described (10). Briefly, various concentrations of staurosporine (Sigma, St. Louis, MO) (0.5, 0.8, 1, 1.2 μM) were tested. Assessment of apoptosis was done by phase-contrast microscopy, fluorescence microscopy of propidium iodide-stained cells, and electron microscopy. Morphological signs of early apoptosis were observed in 40% of the cardiocytes after 3 h of incubation with 0.5 μM staurosporine; after 7 h, 97% of the cells showed signs of more advanced apoptosis. Trypan blue and propidium iodide exclusion indicated membrane integrity. Based on these observations, apoptosis was induced with 0.5 μM staurosporine for 6 h at 37°C in cardiocytes plated at a cell density of 1.2 × 105/75 cm2.

Biotinylation of cell surface proteins
Abs. To evaluate surface expression of SSA/Ro-SSB/La Abs, cell extracts from surface-biotinylated cardiocytes were immunoprecipitated with either human antisera or murine mAbs. Three human antisera were used in these experiments: one contained anti-48-kDa SSA/La, 52- and 60-kDa SSA/Ro Abs (Met), and two sera were from healthy multigravidas with no known autoantibodies (Mo, Hai). Four murine mAbs were used: mAb A2, IgG1 (recognizes 48-kDa SSA/La); mAb A9, IgG1 (recognizes 60-kDa SSA/Ro); W6/32, IgG2a (recognizes the HLA class I glycoproteins HLA-A, B, and C of all alloantigenic species, molecular mass 45 kDa); and MOPC-21, IgG1 (a nonspecific murine mAb).

Surface biotinylation. Surface proteins on apoptotic and nonapoptotic human fetal cardiocytes, cultured in 75-cm2 flasks 4 days after isolation, were labeled with 10 μg/ml N-biotinoyl-ε-aminocaproic acid hydroxysuccinimide ester (Boehringer Mannheim, Indianapolis, IN). Specifically, apoptotic cells were procultured 6–8 h after induction of apoptosis with 0.5 μM staurosporine, when greater than 90% of the cells were apoptotic. Cells in 75-cm2 culture flasks were washed three times with ice-cold HBSS to remove traces of serum proteins from the culture medium. Subsequently, 10 μg/ml N-biotinoyl-ε-aminocaproic acid in ice-cold saline bicarbonate buffer (50 mM NaHCO3, 0.9% NaCl) was added and flasks incubated at 4°C for 20 min. After stopping the biotinylation reaction with ice-cold 50 mM NaHCO3, 0.1 mM biotine-biocarbonate buffer, flasks were washed three times with TBS buffer (50 mM Tris, pH 7.5, 150 mM NaCl). Cells were then incubated for 20 min at 4°C in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1 mg/ml PMSF, and a protease inhibitor cocktail (Complete Mini; Boehringer Mannheim), used as per manufacturer’s instructions. Cells were then scraped off the flasks, transferred to chilled tubes, and centrifuged at 3000 rpm for 10 min. The supernatant was preclarified twice with 50 μl/ml protein A-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). Before immunoprecipitation, a 300-μl aliquot was taken from extracts of apoptotic or control cardiocytes and used as a subsequent SDS-PAGE. The cell extracts were then divided into aliquots and incubated for 1 h at 4°C on a rotating platform with 5 μl of one of the four Abs preparations, as detailed above. Cell extracts corresponding to 3 × 107 cells were used per condition. The protein concentration was equivalent for control and apoptotic cells (2.2 mg/ml), as determined by the BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, IL). Protein A-Sepharose, 50 μl/ml, was then added and immunoprecipitation was conducted overnight at 4°C on a rotating platform. The beads were washed six times with chilled wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 4 mM EDTA-Na2, 0.5% Nonidet P-40, 0.5% deoxycholic acid, sodium salt, 0.1% SDS), eluted, and boiled with reducing SDS sample buffer, then run on 15% high resolution cross-linker acrylamide gels overnight at 7.5 mAsmps until the dye front ran off. The gel was then transferred to nitrocellulose at 40 V overnight. Biotinylated proteins were detected using the bone marrow chemiluminescence blotting kit (biotin/streptavidin; Boehringer Mannheim), according to the manufacturer’s instructions, and filters wrapped in Saran wrap were exposed to x-ray film (Fuji Medical Systems, Stamford, CT).

Scanning electron microscopy studies
To confirm surface expression of SSA/Ro-SSB/La Abs, nonpermeabilized apoptotic and nonapoptotic cardiocytes were incubated with human antisera or murine mAb at a dilution of 1/100 for 45 min in PBS-calcium. One human autoimmune serum contained anti-48-kDa SSA/La Abs alone, as evaluated by ELISA, immunoblot, and immunoprecipitation (Lew); one had 52- and 60-kDa SSA/Ro Abs alone (Dif); and one had 48-kDa SSB/La, and 52- and 60-kDa SSA/Ro Abs (Die). Sera from two healthy multigravidas (Mo, Hai) with no known autoantibodies served as negative controls. The murine mAbs were as described above.

Preparation of cells for scanning electron microscopy. Cardiocytes were prepared on the fourth day after culture to 24-well plates with glass coverslips in the bottom, at a density of 1 × 105 cells/well. After 1 day, apoptosis was induced with 0.5 μM staurosporine, and 5 h later cells were incubated with human sera or murine mAb diluted 1/100 in PBS/0.1 mM CaCl2 (PBS-Ca2+) for 45 min at 37°C, 5% CO2. After washing three times with PBS-Ca2+, cells were fixed with 2% paraformaldehyde in PBS for 20 min at room temperature, washed again, and incubated for 2 h at room temperature with 18 nm goat anti-human (1/10 dilution, AS520 = 2) or 12 nm goat anti-mouse (1/20 dilution, AS520 = 2) colloidal gold alfini-pure IgG (H+L), EM grade (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were washed again and fixed for 1 h at 4°C on a rocking platform with 2% glutaraldehyde in 100 mM cacodylate/100 mM sucrose buffer, pH 7.2, washed three times for 10 min with 100 mM cacodylate/100 mM sucrose buffer, pH 7.2, and dehydrated in increasing concentrations of ethanol. Cells were finally incubated for 1 h in 100% hexamethyldisilazane, air dried, and kept under vacuum until analysis.

Cell-coated coverslips were affixed to aluminum holders using double-sided carbon tape and silver paint, and subsequently coated with a thin layer of carbon to prevent electrical charging when scanning. Samples were examined in a LEO 982 field-emission digital SEM (LEO Electron Microscopy, Thornwood, NY) using 10 kV accelerating voltage and 8 mm working distance. Cell surface morphology was studied by secondary electron imaging with secondary electrons collected by a high-resolution lens secondary electron detector. Backscattered imaging was used in the immunogold studies. The electron backscattering coefficient is a strong function of atomic number, and gold nanoparticles appear bright in a backscattered image. Backscattered electrons emitted from the samples were collected by a solid-state backscattered electron detector (KE Developments, Cambridge, U.K.). All images were recorded and stored digitally. After the images were filtered using DigitalMicrograph software, version
Cocultures of cardiocytes and macrophages

Preparation of affinity-purified Abs and IgG fractions. Abs against 48-kDa SSB/La, and 52- and 60-kDa SSA/Ro proteins were isolated from sera by affinity column chromatography using the respective recombinant proteins (18) coupled to CNBr-activated Sepharose 4B as Ags. Eluted Abs were neutralized with 1 M Tris and tested for specificity by ELISA, immunoblot, and immunoprecipitation of 35S-labeled proteins. Normal human IgG was obtained from normal human serum using the Immunopure (protein A) Purification Kit (Pierce, Rockford, IL), according to the manufacturer’s instructions.

Affinity-purified Abs and normal human IgG were free of endotoxin, as determined by the E-toxate (Limonum amebocyte lysate) assay (Sigma). Affinity-purified Abs did not have rheumatoid factor, as assessed by standard nephelometry.

Culture of human macrophages. Human macrophages were obtained from peripheral blood of normal volunteers, as described (19). In brief, PBMC were isolated by ficoll-hypaque gradient and plated for 2 h in DMEM, in 75-cm² culture flasks. Nonadherent cells were washed out with HBSS, and adherent macrophages were cultured for 4 days in DMEM supplemented with 10% FCS. Subsequently, macrophages were treated with 0.02% EDTA in PBS and transferred to 24-well culture plates at a density of 2 x 10⁵ cells/well.

Opsonization of apoptotic cells. Cultured human fetal cardiocytes, 6 h after induction of apoptosis, were thoroughly washed with HBSS to remove any remaining staurosporine and gently scraped off the culture flasks, resuspended in DMEM (absent FCS) containing affinity-purified Abs or normal human IgG (1.5 µg/ml), and rotated end over end for 30 min at 4°C. After washing three times in DMEM without serum, cells were resuspended in DMEM with 10% FCS and added to macrophage cultures, at a density of 6 x 10⁵ apoptotic cells/well. Culture supernatants were collected 17 h after adding the apoptotic cells, and TNF-α concentration was determined using the high sensitivity Quantikine Immunoassay by R&D Systems (Minneapolis, MN), according to the manufacturer’s instructions.

Phagocytosis of apoptotic cells was verified by light microscopy (data not shown).

Cardiocytes cultured under identical conditions absent the staurosporine served as the nonapoptotic control cells. However, because of their adherence, trypsinization was required. After washing once with DMEM containing 10% FCS to neutralize the trypsin and twice with DMEM (absent FCS), the cells were incubated with serum (from a mother whose child had CHB) containing Abs to all three components of the SSA/Ro-SSB/La complex diluted 1/50 in DMEM.

Results

Immunoprecipitation of surface biotinylated proteins

The initial approach to address the accessibility of autoantigens to circulating maternal Abs utilized immunoprecipitation of surface biotinylated proteins in nonapoptotic and apoptotic cardiocytes. Immunoprecipitation of a biotinylated 48-kDa band was consistently observed in apoptotic cardiocytes incubated with antisera Met, which recognizes 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro (Fig. 1). This band was not detected in cardiocytes that had not undergone apoptosis. No reactivities were seen following immunoprecipitation of either apoptotic or nonapoptotic cells with normal human serum. The intensity of biotin signal in nonimmunoprecipitated cell extracts was similar in control and apoptotic cells, indicating that the efficiency of biotinylation was equivalent in both conditions.

To confirm the identity of the 48-kDa band, immunoprecipitation was done using a murine mAb, A2, which recognizes SSB/La (M48). Equivalent to the results obtained with human antisera Met, a 48-kDa band was observed after immunoprecipitation of the apoptotic but not control cells, confirming the surface expression of SSB/La in the former (Fig. 2). As expected, nonapoptotic and apoptotic cell lysates were immunoprecipitated by a mAb, W6/32, which recognizes the 45-kDa heavy chain of HLA class I molecules. W6/32 served both as a positive control for immunoprecipitation of a known surface protein and facilitated assignment of m.w. No reactivities were seen following immunoprecipitation of either apoptotic or nonapoptotic cells with MOPC-21 (an isotype control mAb).

To further solidify the identity of the SSB/La Ag, cell extracts that did not bind to the protein A-Sepharose beads following incubation with antisera Met or mAb A2 were evaluated by immunoblot. The absence of a 48-kDa band suggested that the SSB/La Ag was depleted from the cell extract as expected. No depletion of 48-kDa SSB/La was seen in cell extracts following immunoprecipitation of surface biotinylated proteins in control and apoptotic cardiocytes. Immunoprecipitates of surface-biotinylated cell extracts from nonapoptotic (lanes 1 and 2) and apoptotic cardiocytes (lanes 3 and 4) are shown to the left. A 48-kDa band is seen in apoptotic cardiocytes (lane 4), but not control cells (lane 2) immunoprecipitated with antisera Met, which recognizes 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro. No bands were seen in control (lane 1) or apoptotic cells (lane 3) after immunoprecipitation with normal human serum (NHS). The two right lanes represent surface-biotinylated cell extracts loaded before immunoprecipitation. The intensity of biotin signal of non-immunoprecipitated cell extracts was similar in control (lane 5) and apoptotic cells (lane 6), indicating that the efficiency of biotinylation was similar in both conditions.

FIGURE 1. Immunoprecipitation of surface biotinylated proteins in control and apoptotic cardiocytes. Immunoprecipitates of surface-biotinylated cell extracts from nonapoptotic (lanes 1 and 2) and apoptotic cardiocytes (lanes 3 and 4) are shown to the left. A 48-kDa band is seen in apoptotic cardiocytes (lane 4), but not control cells (lane 2) immunoprecipitated with antisera Met, which recognizes 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro. No bands were seen in control (lane 1) or apoptotic cells (lane 3) after immunoprecipitation with normal human serum (NHS). The two right lanes represent surface-biotinylated cell extracts loaded before immunoprecipitation. The intensity of biotin signal of non-immunoprecipitated cell extracts was similar in control (lane 5) and apoptotic cells (lane 6), indicating that the efficiency of biotinylation was similar in both conditions.

Immunoprecipitation of surface biotinylated 48-kDa SSB/La using a murine mAb. Immunoprecipitation with mAb A2 (M48) reveals a 48-kDa band in biotinylated cell extracts from apoptotic (lane 4), but not control cardiocytes (lane 1). Immunoprecipitation with a nonspecific mAb MOPC-21 was negative (lane 2, control, and lane 5, apoptotic cells). Immunoprecipitation with mAb W6/32, which recognizes the heavy chain of MHC class I, reveals a band at 45 kDa in both control (lane 3) and apoptotic cardiocytes (lane 6). Biotinylation of surface proteins was equally efficient in control (lane 7) and apoptotic cells (lane 8), as shown by the intensity of biotin signal in cell extracts that were not immunoprecipitated.

FIGURE 2. Immunoprecipitation of surface biotinylated 48-kDa SSB/La using a murine mAb. Immunoprecipitation with mAb A2 (M48) reveals a 48-kDa band in biotinylated cell extracts from apoptotic (lane 4), but not control cardiocytes (lane 1). Immunoprecipitation with a nonspecific mAb MOPC-21 was negative (lane 2, control, and lane 5, apoptotic cells). Immunoprecipitation with mAb W6/32, which recognizes the heavy chain of MHC class I, reveals a band at 45 kDa in both control (lane 3) and apoptotic cardiocytes (lane 6). Biotinylation of surface proteins was equally efficient in control (lane 7) and apoptotic cells (lane 8), as shown by the intensity of biotin signal in cell extracts that were not immunoprecipitated.
immunoprecipitation with normal human serum or W6/32. Of note, these latter immunoblots also demonstrated that 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro were not cleaved during apoptosis (data not shown).

Scanning electron microscopy

Since the interpretation of surface expression, as assessed by biotinylation of immunoprecipitated proteins, might be confounded by minor intracellular uptake of label, scanning electron microscopy was performed utilizing a large number of well-characterized reagents. To restrict labeling to the cardiocyte surface, fixation was performed only after treatment with antisera. Nonpermeabilized apoptotic and nonapoptotic cardiocytes were incubated with experimental human sera or murine mAb, followed by second-stage Abs labeled with gold and observed under a scanning electron microscope. As seen in the backscatter images of Fig. 3, a serum containing 48-kDa SSB/La, and 52- and 60-kDa SSA/Ro (Die) did not bind the nonapoptotic cardiocytes (A). In contrast, gold-labeled particles were readily observed on early apoptotic cardiocytes in which there was surface blebbing but size and morphology were relatively conserved (B). Similarly, there were abundant gold-labeled particles on cells in the later stages of apoptosis, as defined by increased numbers of blebs, decreased size, and rounded shape (C). No gold-labeled particles were seen after incubation with normal human serum (Hai (D) or Mol (data not shown)).

To further elucidate the identity of the bound Ags, antisera with more restricted specificities were used. Binding was observed with a human antiserum containing only 48-kDa SSB/La (Lew) and another recognizing only 52- and 60-kDa SSA/Ro (Dif) (Fig. 4). To unambiguously identify the surface Ags, murine mAb were also used. mAb recognizing 48-kDa SSB/La and 60-kDa SSA/Ro both bound the apoptotic cardiac surfaces (Fig. 5). In all cases, the distribution of gold particles was diffuse with no differences in pattern between the Ags. Cells did not label with the secondary Abs when preincubated with murine isotype control Ab or when primary Ab was omitted (data not shown). For each condition, the number of gold particles was counted in at least seven fields on different cells. The mean ± SEM are summarized in Table I.

**FIGURE 3.** Scanning electron micrographs of cultured human fetal cardiocytes labeled with immunogold attached to a maternal serum containing 48-kDa SSB/La, 52- and 60-kDa SSA/Ro (Die) (A–C), or a normal human serum (Hai) (D). A1–C1, Low power views showing the characteristic morphology of a nonapoptotic (A1), an early apoptotic (B1), and a late apoptotic cardiocyte (C1). A2–C2 and A3–C3, High magnification views of an area on each of these cardiocytes. The same field is studied using two different scanning EM techniques. A2–C2, Demonstrate in detail the topography of the cardiocyte surfaces. A3–C3, A backscatter technique is necessary to contrast the bright metallic signal given by gold particles, present in apoptotic (B3, C3), but not in nonapoptotic cardiocytes (A3). D1–D3, Show a late apoptotic cardiocyte incubated with immunogold and a normal human serum. No gold particles are observed in the backscatter mode (D3). Bars: A1–D1, 5 μm; A2–D2 and A3–D3, 1 μm.
Coincubation of cardiocytes and macrophages

Cardiocytes were coincubated with human macrophages to assess the functional consequences of Ab binding to the surface of apoptotic cardiocytes. The Th1 cytokine, TNF-α, was chosen as a readout of inflammation.

Basal production of TNF-α by the macrophages was 9.7 ± 0.9 SEM pg/ml and decreased to 3.3 ± 0.3 SEM pg/ml after coincubation with apoptotic cells (Fig. 6A). This decrease in basal production was not observed in initial experiments using cardiocytes rendered necrotic after hypotonic lysis (Fig. 6B). Apoptotic cardiocytes preincubated with normal human IgG acted functionally as nontreated apoptotic cells; TNF-α production by the macrophages was 5.7 ± 0.9 SEM pg/ml (Fig. 6A). In contrast, when macrophages were cocultured with apoptotic cardiocytes incubated with affinity-purified Abs to each of the components of the SSA/Ro-SSB/La complex, TNF-α production was increased by 3–5-fold over basal levels and 10–14-fold over that secreted after culture with apoptotic cells alone (specifically, after coculture with cardiocytes opsonized with anti-SSB/La, TNF-α was 35 ± 2.9 SEM pg/ml; with anti-52Ro it was 61.7 ± 10.1 SEM; and with anti-60Ro it was 48.3 ± 9.2 SEM) (Fig. 6A). Nonapoptotic cardiocytes incubated with medium alone or serum containing Abs reactive with 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro did not modify the basal production of TNF-α by the macrophages (Fig. 6C).

Discussion

A role for anti-SSA/Ro-SSB/La Abs in the pathogenesis of CHB is supported by the nearly universal finding of these autoantibodies in mothers of affected children, and by a recently established murine model of CHB in which the offspring of mice immunized with the candidate Ags developed CHB (18). In contrast to neonatal thyrotoxicosis, neonatal myasthenia gravis, or autoimmune thrombocytopenia purpura, in which the fetal target is readily accessible to the extracellular maternal autoantibody, the most difficult problem in CHB has been to explain how intracellularly located SSA/Ro and SSB/La become interactive. Two other puzzling aspects are the incomplete penetrance of disease and the unique fetal susceptibility: CHB develops in only 1–5% of children born to mothers with anti-SSA/Ro-SSB/La Abs (6), recurrence rates are approximately 15% (8), identical twins are often discordant (1), and the maternal heart is not affected. Curiously, the frequency of fetal disease is also unexpectedly low in autoimmune diseases in which adult tissue is affected, Ag is normally surface bound, and Abs cross the placenta, such as myasthenia gravis, thyrotoxicosis, and autoimmune thrombocytopenia (20–22).

Apoptosis is an ideal mechanism to account for the problem of accessibility in the fetal heart and absence of injury in the maternal...
Increased TNF-α and 60-kDa SSA/Ro. Demonstration of surface expression by the SSA/Ro-SSB/La complex: 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro. cardiocytes is indeed bound by Abs to each of the components of the SSA/Ro-SSB/La complex was clearly demonstrated by scanning electron microscopy of the apoptotic cardiocytes. Surface binding was noted in earlier stages of apoptosis, as defined by cell morphology under scanning electron microscopy, and persisted through later stages.

The demonstration of unequivocal Ag-Ab binding at the cell surface supports the hypothesis that maternal Abs to components of the SSA/Ro-SSB/La complex are not simply markers of disease, but play an active part in the pathogenesis of CHB. A molecular explanation for subsequent damage to the specialized cells of the conduction system and working myocardium remains speculative. In this context, a mechanism is envisioned whereby unexpected circumstances convert the physiologic process of apoptosis into one in which an inflammatory component is evoked. Perhaps the unexpected event is opsonization. Apoptotic cells have been regarded as immunosuppressive since internalization of apoptotic cells by phagocytes inhibits the release of proinflammatory cytokines (28, 29). In contrast, phagocytosis of opsonized apoptotic cells has been reported to be proinflammatory (29, 30), an example of which is the observation that ingestion of apoptotic cells bound by anticardioplin Abs results in the release of TNF-α from cultured macrophages (30). Of relevance, two distinct pathways of phagocytosis, each controlled by different intracellular signaling cascades involving Rho GTPases, have been identified (31). Type I follows binding to macrophage Fcγ receptors and is considered to be proinflammatory, while type II, mediated by the complement receptor CR3, is not accompanied by inflammation. Our results support that nonopsonized apoptotic cardiocytes are ingested by macrophages cocultured with nonapoptotic cardiocytes in a serum-dependent manner.

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** *, p vs normal human serum; **, p vs MOPC-2.

heart, since it is a selective process of physiological cell deletion during embryogenesis and morphogenesis that occurs in isolated cells rather than in extensive areas within an organ. Therefore, any disturbance in the noninflammatory mechanism of removal of apoptotic bodies would result in damage to surrounding healthy tissue. Rosen et al. (9) first demonstrated that SSA/Ro-SSB/La ribonucleoproteins translocate to the surface blebs of apoptotic keratinocytes, a seminal observation subsequently corroborated in human fetal cardiocytes (10). Indirect immunofluorescence surface staining of nonpermeabilized cardiocytes with antisera containing Abs reactive with 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro suggested that these Ags not only localize to the surface blebs, but might be displayed in such a way as to be recognized by maternal extracellular Abs. Translocation of other intracellular proteins, calreticulin (23), the 60-kDa heat-shock chaperonin protein (24), and neutrophil myeloperoxidase (25), to the plasma membrane has been reported.

In the study presented herein, two independent techniques provide evidence that the surface of apoptotic cultured human fetal cardiocytes is indeed bound by Abs to each of the components of the SSA/Ro-SSB/La complex: 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro. Demonstration of surface expression by immunoprecipitation of biotinylated proteins was successful for 48-kDa SSB/La only. It has been reported that biotinylation of proteins can abrogate recognition by immunoprecipitating Abs in certain systems (26), and our results may be explained, in part, by hindrance of conformationally dependent epitopes by the biotin compound. Moreover, immunoprecipitation of 52-kDa SSA/Ro is not consistently detected by most laboratories when using cell extracts (27). Surface binding of Abs to each of the components of the SSA/Ro-SSB/La complex are not simply markers of disease, but play an active part in the pathogenesis of CHB. A molecular explanation for subsequent damage to the specialized cells of the conduction system and working myocardium remains speculative. In this context, a mechanism is envisioned whereby unexpected circumstances convert the physiologic process of apoptosis into one in which an inflammatory component is evoked. Perhaps the unexpected event is opsonization. Apoptotic cells have been regarded as immunosuppressive since internalization of apoptotic cells by phagocytes inhibits the release of proinflammatory cytokines (28, 29). In contrast, phagocytosis of opsonized apoptotic cells has been reported to be proinflammatory (29, 30), an example of which is the observation that ingestion of apoptotic cells bound by anticardioplin Abs results in the release of TNF-α from cocultured macrophages (30). Of relevance, two distinct pathways of phagocytosis, each controlled by different intracellular signaling cascades involving Rho GTPases, have been identified (31). Type I follows binding to macrophage Fcγ receptors and is considered to be proinflammatory, while type II, mediated by the complement receptor CR3, is not accompanied by inflammation. Our results support that nonopsonized apoptotic cardiocytes are ingested by macrophages cocultured with opsonized apoptotic (apop) cardiocytes. In A, the results represent the mean of three experiments using different healthy donors of macrophages and different abortuses as the source of the fetal cardiac myocytes. SEM is shown above each bar. In B, the results represent a separate experiment in which macrophages were cocultured with cardiocytes rendered necrotic by hypotonic lysis. In C, macrophages were cocultured with nonapoptotic cardiocytes incubated with medium alone or a serum containing Abs reactive with 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro.
through type II phagocytosis. In contrast, release of the proinflammatory cytokine, TNF-α, by macrophages that have ingested apoptotic cardiocytes bound by anti-SSA/Ro-SSB/La Abs is consistent with type I phagocytosis. These findings suggest that circulating maternal autoantibodies opsonize cells undergoing physiological apoptosis, which then changes otherwise innocent degradation products into proinflammatory stimuli. This cascade could result in damage and ultimately permanent scarring in those tissues with low regenerative capacity.

In summary, the intracellular protein targets, SSA/Ro and SSB/La, are accessible to transplacentally acquired maternal autoantibodies during the process of apoptosis. The results reported in this study support the hypothesis that opsonization of apoptotic cardiocytes by circulating maternal autoantibodies thwarts the normal scavenging function of tissue macrophages during fetal development. Interference with physiologic remodeling might then lead to inflammation. Furthermore, it would appear that the specialized conducting tissue of the human heart is incapable of healing without with fibrotic sequelae.

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References