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Treatment with annexin V increases immunogenicity of apoptotic human T-cells in Balb/c mice

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Abstract

Exposure of phosphatidylserine on the outer leaflet of the cytoplasmic membrane is an early event during apoptotic cell death and serves as a recognition signal for phagocytes. Usually the clearance of apoptotic cells does not initiate inflammation or immune response. We investigated the immune response in Balb/c mice towards apoptotic human T-cells. Animals injected with apoptotic cells showed significantly reduced humoral immune responses, especially Th1-dependent IgG2a titres, compared to controls immunised with viable cells. However, treatment of apoptotic cells with annexin V (AxV) significantly increased the humoral immune response. AxV binds with high affinity to anionic phospholipids and as a result interferes with the phosphatidylserine recognition by phagocytes. Our results indicate that AxV treatment may be used to increase the efficiency of apoptotic cell-based vaccines, e.g. some tumour vaccines. *Cell Death and Differentiation* (2000) **7,** 911–915.

Keywords: apoptosis; engulfment; immunogenicity; annexin V

Abbreviations: AxV, annexin-V; a-huL, anti-human-T-lymphocyte; APC, antigen presenting cells; FA, incomplete Freund's adjuvant; PHA, phytohemagglutinin; PS, phosphatidylserine

Introduction

During the course of pathological conditions cells may die through necrosis involving disruption of membrane integrity, subsequent swelling, and finally cellular lysis. In contrast, cells undergoing apoptosis retain their membrane integrity and are scavenged before they release potentially dangerous contents into the microenvironment. As part of the apoptotic death program cells undergo rapid surface changes such as modification of carbohydrates and exposure of anionic phospholipids especially phosphatidylserine (PS). The latter response is caused by downregulation of the ATP-dependent aminophospholipid translocase, which specifically transports aminophospholipids from the outer to the inner leaflet of the cytoplasmic membrane. Furthermore, a non-specific lipid flipsite, termed scramblase, is activated resulting in an accelerated PS flip-flop.[1] It is well established that PS serves as recognition signal for the clearance of apoptotic cells.[2–5] In a recent publication it was shown that preincubation of apoptotic lymphocytes with AxV efficiently blocked the uptake of apoptotic cells by murine peritoneal macrophages, macrophages of the mouse J774 cell line and bone marrow macrophages.[6] In addition, this study demonstrated that AxV generally leads to a strong inhibition of apoptotic cell uptake by both activated and non-activated macrophages.[6] Furthermore, it was demonstrated that redistribution of PS both on phagocyte and prey is involved in the engulfment of apoptotic cells.[7] This paper confirmed the inhibition by annexin V of apoptotic cell uptake.[7] In addition, annexin V was shown to decelerate apoptosis in CEM cells by its Ca^{2+} channel activity.[8]

Usually engulfment of apoptotic cells induces neither inflammation[5] nor an immune response.[9] In direct contact to plasma, apoptotic cells may exert procoagulatory and under certain conditions, proinflammatory effects[10–12] due to de-encryption of tissue factor.[13] However, anti-inflammatory and immunomodulatory effects of apoptotic cells on monocytes/macrophages usually dominate, for instance through the interaction of apoptotic cells with CD36 on phagocytes via thrombospondin.[14,15]

Lectin-like molecules, the vitronectin receptor (CD51/CD61), thrombospondin, CD36,[5] as well as CD14[16,17] have all been described to be receptors which recognise surface changes on apoptotic cells. CD14 seems to be required for phagocytosis of lymphocytes and necessary for phagocytosis of lipid-symmetric erythrocytes both by non-activated and activated macrophages.[1]

Since phagocytosis of apoptotic lymphocytes by macrophages is stereospecifically inhibited by phosphatidyl-L-serine liposomes, a specific receptor may be involved in PS recognition.[18] This PS-receptor has not been clearly defined yet, however, CD14, CD68, CD36[19] have been proposed as candidates. In addition, β2-glycoprotein, the annexins and gas-6 may serve as adaptor proteins.[20] Furthermore, a novel receptor defined by monoclonal antibodies elicited by immunisation with TGF-β- and β-glucan-stimulated macrophages was reported to mediate PS-dependent uptake of apoptotic cells.[21] (Presented at the 7th Bertine Koperberg Conference on 'Apoptosis and Autoimmunity', Nijmegen, June 1999.)

We have recently shown that animals injected with apoptotic human T-cells in comparison to controls injected with viable cells showed significantly decreased humoral immune responses against human T-cells.[22] As we demonstrate here, apoptotic cells are a poor immunogen to elicit Th1-dependent IgG2a antibodies. Furthermore, Ronchetti and colleagues recently observed that immunisation with apoptotic cancer cells induced drastically decreased cytotoxic T-cell responses compared to immunisation with living, growth arrested cells.[23] These reports indicate that engulfment phagocytosis of apoptotic cells does not lead to efficient antigen presentation and activation of T and B lymphocytes. It is possible that rapid engulfment phagocytosis of apoptotic cells by macrophages may prevent uptake and efficient presentation of apoptotic cell-derived antigens by dendritic cells. Together with an increased production of IL-10 by monocytes after encountering apoptotic cells, these results may explain the poor efficiency of those cancer vaccines containing apoptotic cells.[14,23] Therefore, we speculate that surface changes during apoptosis direct apoptotic cells towards phagocytosis by monocytes/macrophages, without induction of either inflammation or an immune response.

AxV which binds with high affinity PS on dying cells efficiently blocks the uptake of apoptotic cells into macrophages *in vitro*.[6,24] To investigate whether the immunogenicity of apoptotic cells can be increased by interfering with PS recognition by phagocytes, we incubated xenogeneic apoptotic cells with AxV prior to injection into mice. AxV treatment markedly increased the immunogenicity of xenogeneic apoptotic cells. Therefore, we concluded that AxV may be useful to increase the efficiency of those cell based vaccines containing apoptotic cells.

Results

AxV specifically binds to PS with a high affinity in a Ca^{2+}-dependent manner.[25–27] Therefore, AxV is commonly used to detect PS on surfaces of apoptotic cells.[28] In addition, AxV has been shown to inhibit phagocytosis of apoptotic cells by human[24,29] and mouse macrophages.[6] Similarly, in coculture experiments of UV-B irradiated murine macrophages with viable thioglycollate elicited murine macrophages we observed that the addition of chicken AxV resulted in a markedly increased amount of uningested apoptotic cell material (data not shown).

Figure 1 shows the AxV binding of viable *vs* apoptotic human T-cells used for primary immunisation of Balb/c mice. Approximately 70% of the irradiated T-cells exposed PS as analyzed by AxV-FITC binding, whereas only 10% of the non-irradiated cells were stained with AxV-FITC. Using conventional light microscopy immediately before injection we observed that at least 85% of the UV irradiated cells excluded trypan blue. Therefore, most of the irradiated T-cells displayed an apoptotic phenotype at the time of injection.

In preceding immunisation experiments we compared the human T-cell lines used for immunisation with HUT78 cells as targets for detection of murine antibodies against human T-cells by flow cytofluorometry. No difference was observed between both target cell types (data not shown). In this study we employed HUT78 for quantification of murine antibodies against human T-lymphocytes (a-huL), since they can be grown continuously and independently of restimulation cycles.

To further analyze the reduced immunogenicity of apoptotic cells and to investigate whether masking of PS by AxV can restore the humoral immune response to apoptotic cells, we immunised Balb/c mice with viable *vs* apoptotic human T-cells. Highly immunogenic xenogeneic cells were used to exclude the possibility of direct antigen presentation by the injected cells to recipient T-cells.[30] After primary immunisation of mice with human T-cells, the a-huL

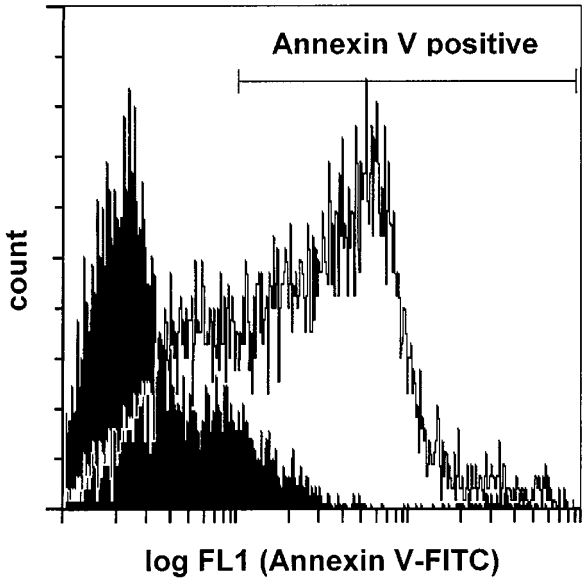

Figure 1 AxV-FITC binding of viable versus apoptotic human T-cell lines used for immunisation of Balb/c mice. At the time of injection more than 85% of the cells excluded trypan blue. Staining with FITC labelled AxV revealed PS exposure on 70% of the UV-B irradiated apoptotic cells (white), whereas only 10% of the unirradiated viable cells (black) exposed PS at the time of injection

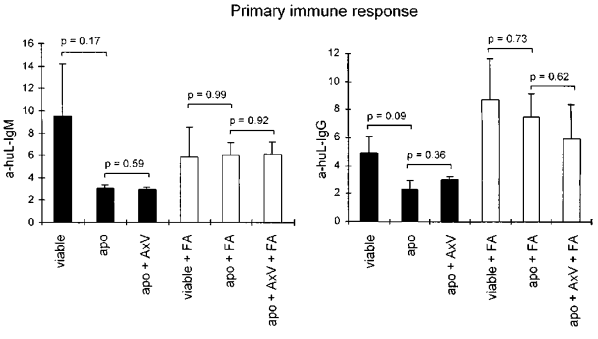

Figure 2 Influence of AxV on the primary humoral immune response. Anti-human-lymphocyte antibodies (left, IgM; right, IgG) in mice which had been injected once intraperitoneally with viable human T-cells (viable), apoptotic T-cells (apo) and AxV treated apoptotic T-cells (AxV) in the absence (black bars) or presence (white bars) of FA. P-values were calculated using Students' *t*-test. The Y-axis shows immunofluorescence values in arbitrary units

antibody titres were low (Figure 2). Animals which had received apoptotic cells displayed reduced a-huL-titres compared to mice injected with viable cells. Co-injection with FA of either viable or apoptotic cells modestly increased a-huL-IgG titres (Figure 2, right).

After booster injection a-huL-IgG-titres (Figure 3, right) were markedly lower in mice which had received apoptotic cells than in mice injected with viable cells (mean fluorescence (MF)-IgG: 32 ± 7.3 and 126 ± 6.3, respectively; $P<0.0001$). Treatment of apoptotic cells with AxV significantly increased the a-huL-IgG titres (MF-IgG: 89 ± 10; $P=0.0015$). The a-huL-IgM-titres (Figure 3, left) were only slightly reduced in mice injected with apoptotic cells compared to mice injected with viable cells (MF-IgM: 6 ± 1.4 and 11 ± 3.2, respectively; $P=0.036$). Therefore, treatment of apoptotic human lymphocytes with AxV largely restored their immunogenicity (Figure 3).

Co-injection with FA of either viable or apoptotic cells modestly increased a-huL-IgG titres (Figure 3, right), whereas a-huL-IgM-titres were only slightly increased. The a-huL-IgG titres were significantly decreased in mice which had received apoptotic cells (MF-IgG: 70 ± 3.6 compared with 156 ± 9.9; $P=0.004$). Treatment of apoptotic cells with AxV led to significantly increased a-huL-IgG-titres (MF-IgG: 138 ± 4.3 compared to 70 ± 3.6; $P<0.0001$).

Figure 4 shows that the a-huL-IgG1 and IgG2a titres in mice which had received apoptotic cells were significantly reduced compared to mice injected with viable cells (MF-IgG1: 13 ± 1.1 and 21 ± 2.2 respectively; $P=0.010$; MF-IgG2a: 5.6 ± 2.0 and 26 ± 3.6 respectively; $P=0.0007$). While the a-huL-IgG1 titres in mice injected with AxV treated apoptotic cells were only marginally increased, the a-huL-IgG2a titres were more than doubled by AxV treatment, however, this result did not reach statistical significance ($P=0.081$). Preabsorption of murine sera with AxV did not reduce the a-huL-titres, thereby excluding the possibility that AxV antibodies are responsible for the increased a-huL-titres in mice immunised with AxV treated cells (not shown).

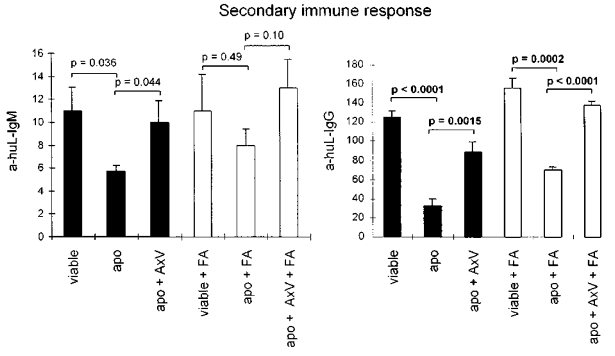

Figure 3 Influence of AxV on the secondary humoral immune response. Anti-human-lymphocyte antibodies (left, IgM; right, IgG) in mice injected twice intraperitoneally with viable human T-cells (viable), apoptotic T-cells (apo) and AxV treated apoptotic T-cells (AxV) in the absence (black bars) or presence (white bars) of FA. *P*-values were calculated using Students' *t*-test. Statistically highly significant differences are displayed in bold face letters ($P<0.01$). The Y-axis shows immunofluorescence values in arbitrary units

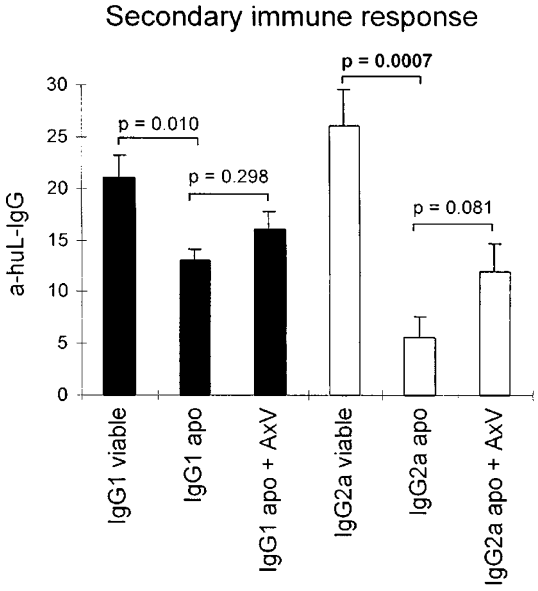

Figure 4 Influence of immunisation in the presence of AxV on IgG subclass titres. Anti-human-lymphocyte-IgG1 (black bars) and IgG2a titres (white bars) in mice which had been injected twice intraperitoneally with viable human T-cells (viable), apoptotic T-cells (apo) and AxV treated apoptotic T-cells (AxV). *P*-values displayed according to Students' *t*-test. Statistically highly significant differences are displayed in bold face letters ($P<0.01$)

Discussion

Reduced immune responses against apoptotic cells have been observed in an inbred mouse model,[9] in Balb/c mice immunised with xenogeneic apoptotic lymphocytes[22] and in C57BL/6 mice after immunisation with apoptotic tumour cells.[23–31] Furthermore, transfusion of viable blood mononuclear cells has been reported to induce IgM, IgG1, and IgG2a anti-donor antibodies resulting in a suppression of subsequent delayed type hypersensitivity reactions, while apoptotic cells led to neither alloimmunisation nor immunosuppression.[9]

Since alloantigens can be presented to recipient T-cells either directly by donor antigen presenting cells (APC) or indirectly by recipient APC, the mechanism of antibody induction in the murine alloimmunisation experiment can not be defined precisely. To exclude direct presentation, which would be dependent on costimulatory signals from donor APC, we used xenogeneic T-cell lines as the immunogen. In this case a direct presentation appears highly unlikely and an immune response should be exclusively dependent on indirect antigen presentation by murine APC after phagocytosis of the human T-cells. One may speculate that in this system the reduced immunogenicity of apoptotic cells is due to a failure of murine APC to activate T-cells after engulfment of apoptotic cells. In syngeneic and presumably many allogeneic murine systems both direct and indirect antigen presentations are basically possible and could not be clearly distinguished.[30]

We hypothesise that the engulfment of apoptotic cells by macrophages normally engages a processing pathway which does not result in efficient antigen presentation

and/or costimulation with consecutive T-cell activation. To investigate this hypothesis we incubated apoptotic cells with AxV in order to mask PS and thereby to interfere with the PS dependent phagocytosis. The calcium-dependent inhibition of phagocytosis of apoptotic cells by AxV was first described for smooth muscle cells.[24] In recent publications it was shown that AxV generally leads to a marked inhibition of the uptake of apoptotic cells by both activated and unactivated macrophages.[6,7] Treatment of apoptotic cells with AxV increased their immunogenicity, suggesting that they were able to encounter a pathway leading to both antigen presentation and delivery of costimulatory signals. We speculate that AxV interfered with the PS recognition and the highly efficient engulfment of apoptotic cells by macrophages. Therefore, the uptake and presentation of apoptotic cell material by dendritic cells might be favoured. Recently it was shown that dendritic cells are able to acquire antigens from apoptotic cells[23,30–32] and elicit a class I-restricted CTL response.[33] However, in the presence of macrophages the CTL response was abrogated. The authors suggested that macrophages efficiently engulfing apoptotic cells may degrade the antigen.[33] Another explanation for the effect of annexin V on the immunogenicity of apoptotic cells is that under certain circumstances annexin V is able to delay apoptotic cell death by increasing the intracellular Ca^{2+} concentration.[8] However, there are other reports linking the increase of intracellular Ca^{2+} concentration with augmentation of apoptosis.[34]

Although a decreased immune response against apoptotic cells could be observed for IgG1 as well as IgG2a, the decrease was more pronounced for the Th1-dependent IgG2a subclass. In addition, AxV treatment predominantly increased the IgG2a response. This argues for a preferential inhibition of the Th1-like responses. Interestingly, we have observed earlier that the presence of apoptotic cells in monocyte cultures can shift the Th1 cytokine secretion pattern towards Th2.[14] An alternative explanation for the increased immunogenicity of AxV treated apoptotic cells would be a disruption of the cytoplasmic membrane by AxV resulting in necrosis and release of immunostimulatory or inflammatory mediators,[9] thereby providing a yet undefined 'danger' signal for the APC.[30,31] However, *in vitro* AxV does not cause cytoplasmic membrane leakage as shown by dye exclusion.[28] Furthermore, AxV treatment did not lead to an increased immunogenicity of viable human T-cells (not shown).

Our data may have implications for the development of vaccines which are based on apoptotic cells. For instance, cellular tumour vaccines usually display a poor immunogenicity if the cancer cells are irradiated before injection.[35,36] Thereby, irradiation-induced apoptosis may result in a non-inflammatory engulfment by macrophages and, in addition, an anti-inflammatory effect of apoptotic cells on human monocytes/macrophages.[14,15] Based on our immunisation experiments with xenogeneic apoptotic cells we assume that treatment with AxV may also increase the immunogenicity of irradiated tumour vaccines.

Taken together our data show that xenogeneic apoptotic cells are poor inducers of humoral immune responses, especially Th1-dependent Ig2a antibodies. Furthermore, we have demonstrated that the immunogenicity of apoptotic cells can be largely restored by AxV treatment.

Materials and Methods

Animals

Balb/c mice were obtained from Charles River Wiga (Sulzfeld, Germany). Six-week-old female mice were used in the experiments.

Cell culture and induction of apoptosis

Human T-cell lines were established from peripheral blood mononuclear cells (PBMC) using phytohemagglutinin (PHA) activation and IL-2. T-cells were maintained and expanded in RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 30% CG medium (Vitromex, Vilshofen, Germany), 5% heat inactivated foetal bovine serum (Gibco, Eggenstein, Germany), gentamycin (100 μg/ml) and recombinant human IL-2 (100 U/ml) (Eurocetus, Frankfurt, Germany) at 37°C and 5% CO_2. Every 3 weeks T-cells were restimulated using PHA (1 μg/ml) in the presence of irradiated (30 Gy) heterologous PBMC.

Immunisation

The cloning and the isolation of chicken AxV used in our studies was performed as described elsewhere.[26] In one set of experiments Balb/c mice were assigned randomly to three treatment groups (n=6), intraperitoneally injected with viable cells, apoptotic cells or AxV-treated apoptotic cells, respectively. In another set of experiments the cell suspension was mixed with FA (140 μl/ml) immediately before injection (n=4). Seventeen days after the primary immunisation all animals received a booster injection identical to their primary immunisation.

After apoptosis induction by UV-B irradiation (180 mJ/cm^2) human T-cells were cultured in medium without IL-2 for another 20 h. The cells were then harvested and washed twice with Ringer's solution. One million cells/ml were incubated in Ringer's solution in the presence or absence of AxV (1.2 μg/ml) on ice for 30 min. Each Balb/c mouse was then injected intraperitoneally with 5×10^5 cells suspended in 500 μl Ringer's solution without prior washing. At the time of injection more than 90% of these T-cells displayed an apoptotic phenotype according to microscopic evaluation and by propidium iodide staining in the presence of Triton X-100. More than 85% of the cells excluded trypan blue and approximately 70% of these cells had exposed PS as measured by AxV binding. Therefore, most of the cells displayed an early apoptotic phenotype.[37] Non-irradiated control cells were cultured in the presence of IL-2, washed twice and resuspended in Ringer solution 30 min before injection. For the detection of PS exposure, 10^5 cells were stained with 0.5 μl AxV-FITC (Boehringer, Mannheim, Germany) in 500 μl Ringer's solution on ice for 30 min and analyzed by cytofluorometry.

Analysis of sera by flow cytofluorometry

Ten days after each injection, murine sera were collected and stored at -20°C. Preimmune sera and sera from mice injected with Ringer's solution served as negative controls. Murine a-huL were quantified by indirect immunofluorescence using either viable or apoptotic HUT78 cells as well as the T-cell lines used for immunisation. Cells (1.2×10^5) were incubated with 5 μl mouse serum (diluted 1 : 10 with PBS

containing 1% BSA and 0.1% NaN_3) for 1 h at 4°C. After washing the cells twice with FACS-PBS 5 μl FITC labelled anti-mouse-IgG, anti-mouse-IgM, anti-mouse-IgG1 or anti-mouse-IgG2a serum was added for 30 min at 4°C. The bound fluorescence was measured using an EPICS XL^{TM} cytofluorometer (Coulter, Hialeah, USA). All data presented in the figures were obtained using viable HUT78 as targets.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. and statistical significance was analyzed by one-tailed Students' *t*-test for unpaired data (InStat 2.01, GraphPad Software, San Diego, USA).

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