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Molecules and Cells

Downregulation of MHC Class II Expression by Oxidant-induced Apoptosis in EBV-transformed B-Cells

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The expression of MHC class II molecules is actively regulated upon various cellular stimuli. Since apoptosis is an inducible cellular process, it was asked whether cells undergoing apoptosis would also modulate their expression of class II molecules. Using an EBV-transformed B-cell line, the cell surface expression of HLA-DR molecules was analyzed by fluorescence-activated flow cytometry on normal and oxidant-treated apoptotic cells. A rapid and continuous decrease in HLA-DR expression was observed in apoptotic cells. RNA analysis and semiquantitative RT-PCR of cytoplasmic β-actin mRNA showed that apoptotic cells contain partially degraded RNA and much lower amounts of β-actin mRNA. Nevertheless, when compared after normalization of intact mRNA amounts, the HLA-DRB mRNA signals were of similar strength in normal and apoptotic cells as determined by semiquantitative RT-PCR. Thus, the decrease in the number of class II molecules during apoptosis underlies no specific program for downregulation of HLA-DRB mRNA transcription but is due to a nonspecific degradation of RNA molecules accompanied by cell death.

Keywords: Apoptosis; Competitive Reverse Transcription-PCR; H_2O_2; Internal Control Plasmid; MHC Class II.

Introduction

MHC class II molecules are cell surface glycoproteins involved in the transport and presentation of exogenous peptide antigens to CD4 T-lymphocytes (Miller and Sant, 1995). The expression of MHC class II molecules is largely restricted to cells termed professional antigen-presenting cells but they can also be induced on otherwise nonexpressing cells by activation over cell surface receptors (Gansbacher and Zier, 1998; Zier *et al*., 1989) or IFN-γ treatment (Rohn *et al*., 1996). The molecular mechanisms behind the induction and expression of MHC class II molecules have been extensively studied, and it is now widely accepted that the modulation of class II expression is mainly controlled on a transcriptional level (Glimcher and Kara, 1992). Furthermore, in a recent report, the class II transactivator (CIITA) has been assigned to be the major, if not sole, control factor for the quantitative regulation of class II transcription (Otten *et al*., 1998). Nevertheless, little is known about the signal molecules upstream to CIITA or about the direct cellular signals leading to the transactivation of MHC class II transcription. While cell activation leads in many cases to the upregulation of MHC class II expression, some viral products (Maudsley and Pound, 1991) as well as cytokines such as IL-10 (Koppelman *et al*., 1997) or TNF-α (Konig *et al*., 1997) actively down-modulate the cell surface expression of class II molecules.

Induction of apoptosis is an active cellular response, which involves the initiation of a large cascade of intracellular signals leading to the induction of new genes (Wang *et al*., 1997) and activation of various proteases (Villa *et al*., 1997) and nucleases (Enari *et al*., 1998). While there have been extensive descriptions of the morphological and biochemical changes during the apoptotic process, only little is known about the alterations on the cell surface. The disturbance of the asymmetric organization of membrane lipids and the resulting exposure of anionic lipid molecules, such as phosphatidylserine (Martin *et al*., 1995), is one of the few events that is known to happen at the cell membrane by apoptosis. In the case of modulation of cell surface protein expression, there is a remarkable lack of reports. Only recently, a few reports have been presented on the alterations of some cell-type specific marker proteins in apoptotic neutrophils (Dransfield *et al*., 1994; Homburg *et al*., 1995), erythroleukemia

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cells (Maccarrone *et al.*, 1996), peripheral T cells (Renno *et al.*, 1996) and thymocytes (Kishimoto *et al.*, 1995); however, cell surface changes in apoptotic B cells have still to be reported, and, especially in regard to the expression of MHC class II molecules, almost nothing is known. Since MHC class II molecules are important regulators of the cellular immune response, it is of great interest to examine in which way these molecules are modulated upon induction of apoptosis. In particular, the expression of class II molecules on B lymphocytes, which represent a major source of MHC class II positive cells in the immune system, would serve as an excellent subject for analysis of the dynamic regulation of class II expression during apoptosis. In the present study, the expression of MHC class II molecules was determined and compared between normal and apoptotic EBV-transformed human B lymphocytes using fluorescence-activated flow cytometry. Furthermore, to analyze the mechanism by which the expression of class II molecules is modulated, the HLA-DR β-chain mRNA signal was semiquantitatively measured using internal controls by competitive RT-PCR (Khan *et al.*, 1996).

Materials and Methods

Cell culture and induction of apoptosis The EBV-transformed B-cell line, Wa (Katagiri *et al.*, 1979), was a kind gift from Dr. Keisuke Sato, Asahikawa Medical College, Japan. Cells were maintained in RPMI-1640 media supplemented with 10% FBS (Life Technologies, Grand Island, NY, USA) in a humidified incubator at 5% CO_2 atmosphere. Depending on the experimental design, the cells were cultured in the presence of variable concentrations of H_2O_2 for different times. Cell viability was determined by trypan blue exclusion tests.

DNA fragmentation assay Analysis of DNA fragmentation by apoptosis was performed after the protocol described by Homburg *et al.* (1995). Briefly, for a single assay, 1×10^6 cells were harvested and resuspended in cell lysis buffer (0.5% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) and treated with proteinase K (0.5 mg/ml) at 56°C for 2 h. After an additional RNase digestion step with 0.25 mg/ml RNase A (Roche Molecular Biochemicals, Mannheim, Germany) at 37°C for 1 h, an aliquot of the whole cell lysate was loaded in the well of a 2% TAE-agarose gel and analyzed by gel electrophoresis.

Immunofluorescence staining and flow cytometry Normal or oxidant-induced apoptotic cells were harvested from culture and washed once in PBS/0.1% BSA/0.05% Na-azide before immunostaining. MHC class II molecules were detected with the anti-HLA-DR murine monoclonal antibody L243 (Schackelford *et al.*, 1981), and cell surface-bound antibodies were detected with FITC-conjugated goat anti-mouse IgG antibodies (DAKO A/S, Glostrup, Denmark). As a negative control the isotype-matched mouse monoclonal antibody, HAM-19 (Park *et al.*, 1998a), which binds to *E. coli* maltose-binding proteins, was used. Flow cytometric analysis was performed with a FACScan™ (Becton Dickinson, Mountain View, CA, USA), and the data were analyzed using the LYSYS software.

RNA isolation and reverse transcription Whole cellular RNA was isolated by guanidium thiocyanate/phenol/chloroform extraction as originally described by Chomzynski and Sacchi (1987). Purified RNA was analyzed in a MOPS-buffered 1.5% agarose gel containing formaldehyde as denaturing agent and then visualized after EtBr-staining under UV light. For cDNA synthesis, 1 μg of whole RNA was taken and reverse transcribed in a 40 μl reaction using SuperScript™ II enzyme (Life Technologies) and oligo-dT_{15} primers (Promega, Madison, WI, USA).

Generation of an internal control plasmid for determination of the cytoplasmic β-actin mRNA A 400-bp PCR fragment was amplified from normal Wa-cell cDNA using the cytoplasmic β-actin specific primer pairs (GenoTech, Taejon, South Korea) as shown in Table 1. To generate a DNA fragment which competes with the actual cellular cDNA for the β-actin primers, but that can be discriminated by its different size, the 400-bp PCR fragment was digested with the restriction enzyme *Hpa*II (Boehringer Mannheim, Mannheim, Germany), by which an internal 100-bp fragment was deleted from the PCR product. Ligation of this fragment mixture and subsequent PCR amplification with the β-actin primers resulted in the identification and isolation of a 300-bp PCR fragment, which was further subcloned into a pGEM-T vector (Promega). The resulting plasmid, termed pGEM-actin/S, served as an internal standard for semiquantitative RT-PCR analysis of cytoplasmic β-actin mRNA signals.

Generation of an internal control plasmid for determination of the HLA-DR β-chain mRNA The internal control plasmid for competitive PCR analysis of the HLA-DR β-chain mRNA was constructed from a previously described vector, the pQE-Ex2/B1*0405 which contains a 261-bp fragment from the β1-region of the HLA-DRB1*0405 cDNA (Park *et al.*, 1998b). Digestion of this vector with the restriction enzyme *Pst*I resulted in the excision of a 102-bp fragment with simultaneous opening of the vector. To increase the size of the competitor fragment, a 287-bp DNA fragment predigested with *Nsi*I on both ends was subcloned into the *Pst*I site of the vector. This insert was generated by PCR amplification of the 3′-downstream region of the 261-bp DR β-chain region within the original pQE-Ex2/B1*0405 vector using the primer pairs "DRB-mid-*Nsi*I" and "pQE-reverse-*Nsi*I" (see Table 1). The resulting plasmid, termed pQE-DRB/S, generated a 126-bp larger fragment over the PCR product from cellular cDNA

Table 1. Nucleotide sequences of the PCR primers.

Primers	Sequence
β-Actin sense	5′-acatggagaaaatctggcac-3′
β-Actin antisense	5′-ctccttaatgtcacgcacg-3′
DRB-mid-*Nsi*I	5′-ccaatgcatgcgcttcgacagcgac-3′
pQE-reverse-*Ns*i	5′-ggtatgcatgttctgaggtcattactgg-3′
HLA-DRB1	5′-gtttcttggagcaggttaaac-3′
HLA-DRB2	5′-cgctgcactgtgaagctctc-3′

when PCR was performed using the HLA-DRB1*0405 specific primer pairs, DRB-1 and DRB-2 (see Table 1).

Competitive RT-PCR Using reverse-transcribed cDNA, all PCR were performed after the following protocol: 2 min denaturation at 96°C, then 36 cycles of a 45 s denaturation step at 95°C, 1 min annealing at 55°C, 1 min elongation at 72°C, and a final elongation step for 5 min at 72°C. PCR was performed on a GeneAmp 2400 thermal cycler (Perkin Elmer, Foster City, CA, USA) using 1 unit of *Taq* polymerase (Promega) per reaction. For primer competition, serially diluted internal controls were coamplified in a series of PCR either for the detection of cytoplasmic β-actin or for HLA-DRB mRNA.

The PCR products were resolved in an 1.5% TAE-agarose gel, and the signal intensity of each band was quantified by densitometric analysis of the EtBr-stained DNA using a CCD-camera linked to an image analyzer system (Vilbert Lourmat, Marne La Valee, Cedex, France) and the accompanying software, BIO-1D. The plasmid numbers used as internal standards were calculated using the following equation (Dumoulin *et al.*, 1997): molecule number = amount of internal standard (g) × Avogadro's number (molecules/mol)/mass of internal standard (g/mol). Using this equation, it was calculated that 1 μg of a 1,000-bp DNA would correspond to 1.52 pmol, which in turn would correspond to 9.1×10^{11} molecules. In practice, the number of particular molecules which are present in the reverse-transcribed mRNA was estimated by the number of control plasmid copies which was required to reach the equivalence point in the PCR reaction, where the signal intensity of the competitor/target ratio was 1. For further review of the principles of competitive, semiquantitative PCR, see Piatak *et al.* (1993) and Dumoulin *et al.* (1997).

Results

Oxidant-induced apoptosis Induction of apoptosis by oxidants (H_2O_2) was performed as previously described by Lee and Shacter (1997). Briefly, Wa cells were harvested, counted, and re-plated to a concentration of 2×10^5 cells/ml in RPMI-1640 media into 100 mm culture dishes. The cells were then incubated for 24 h with different concentrations of H_2O_2, and then the viability was determined by the trypan blue exclusion method. As shown in Fig. 1A, overnight treatment with H_2O_2 to a final concentration of 1 mM was sufficient to induce cell death in most of the cells. This condition was then used for all further experiments. Phase-contrast microscopy of the oxidant-treated cells showed plasma-membrane blebbing and other characteristic phenotypic differentiation for apoptosis (data not shown). Conformation on the apoptotic state of the H_2O_2-treated cells was further given by analysis of the genomic DNA. As shown in Fig. 1B, chromosomal DNA from H_2O_2-treated, but not that from normal Wa cells, generated the typical 200-bp DNA ladder, which is usually regarded as a hallmark for cells undergoing apoptosis (Walker *et al.*, 1993).

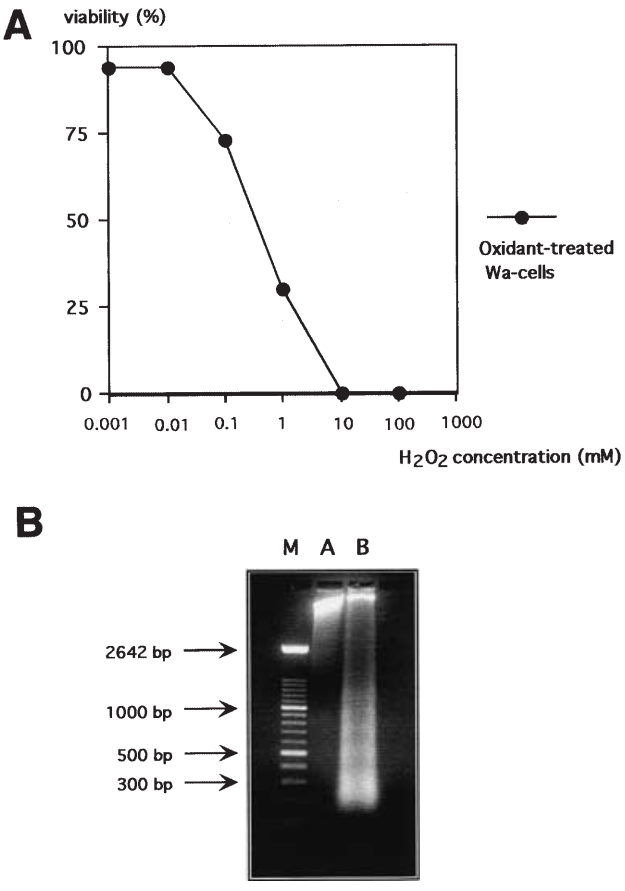

Fig. 1. Cell viability of EBV-transformed Wa cells treated with different concentrations of H_2O_2. The day before analysis, cells were seeded at a density of 2×10^5 cells/ml in serum supplemented RPMI-1640 media. H_2O_2 was added as indicated, and the next day, the numbers of live and dead cells were determined by trypan blue exclusion. **A.** The viability (%) is defined as the number of live cells divided by the number of total cells counted. For each concentration, 500 cells were counted. **B.** Agarose gel shows the status of isolated genomic DNA from either cells cultured under normal condition or those incubated overnight in the presence of 1 mM H_2O_2. Genomic DNA was isolated from normal (**A**) and oxidant-treated (**B**) Wa cells and analyzed in a 2% TAE-agarose gel. The 100 bp-ladder molecular-weight marker (DNA molecular weight marker XIV, Boehringer Mannheim) is shown in lane M.

Flow cytometric analysis of apoptotic cells The effect of H_2O_2 on changes of cell morphology and surface molecule expression was analyzed by fluorescence-activated flow cytometry. As shown in Fig. 2, up to 24 h after oxidant treatment, no severe alterations in cell volume and cell size were observed, which indicated the overall conservation of the cell structure. Analysis of HLA-DR cell surface expression was performed using the HLA-DR-specific monoclonal antibody L243, by which, as expected, a high level expression of MHC class II molecules was observed on normal Wa cells. On the other hand, a continuous decrease of class II

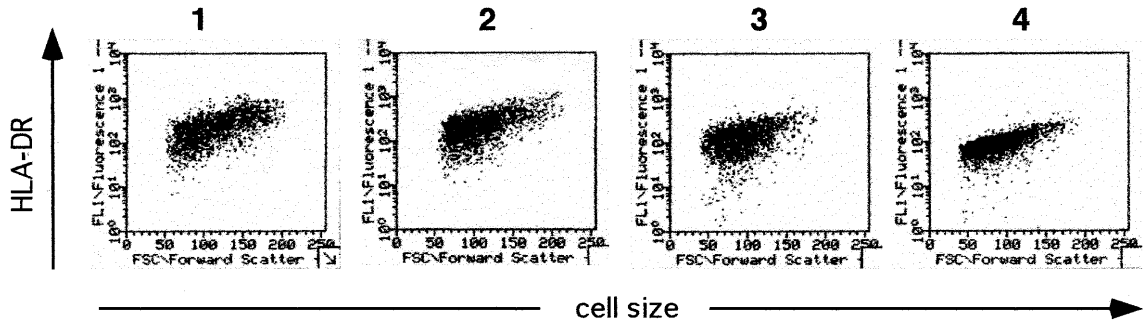

Fig. 2. Flow cytometric analysis of normal and apoptotic EBV-transformed B-cell MHC class II expression on Wa-cells treated with 1 mM H_2O_2 at different times was determined using the HLA-DR specific monoclonal antibody L243. Dot-plot diagrams show the cell size (forward scatter) on the horizontal axis plotted against HLA-DR staining on the vertical axis. (1). Wa cells maintained in RPMI-1640 media with 10% FBS; (2). Wa cells incubated for 6 h with 1 mM H_2O_2; (3). Wa cells incubated for 24 h with 1 mM H_2O_2; (4). Wa cells incubated for 48 h with 1 mM H_2O_2.

molecule expression was induced when the molecules were maintained in the presence of 1 mM H_2O_2 for prolonged times.

Analysis of total RNA from normal and apoptotic Wa cells Whole cellular RNA from apoptotic and normal Wa cells was resolved in an 1.5% MOPS-buffered agarose gel with formaldehyde. In both cases, the 28S and 18S ribosomal RNA were clearly observable. Judging from the signal strength of the ribosomal RNA in both cases, the absolute amount of whole RNA seemed to be comparable between normal and apoptotic cells. Nevertheless, the presence of some distinct additional bands in the RNA from apoptotic cells as well as a stronger smearing background (Fig. 3) indicated a partial degradation of the RNA by induction of apoptosis.

Semiquantitative RT-PCR of cytoplasmic β-actin and HLA-DRB mRNA For the semi-quantitative analysis of β-actin mRNA in oxidant-treated Wa cells, competitive PCR was performed with reverse-transcribed mRNA of both apoptotic and normal Wa-cells. Each 1 μl of the RT reaction was used for one PCR sample. The competitor molecules (Fig. 4) were added to different concentrations in a serial dilution, the starting concentration being set depending on the origin of the cDNA. Since a relatively high copy number of intact β-actin mRNA was expected in normal Wa cells, the

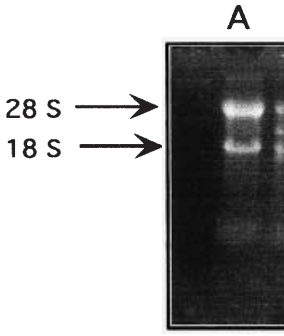

Fig. 3. Isolation of whole RNA from apoptotic and normal Wa cells. Whole RNA was isolated from normal A and oxidant treated B Wa cells and analyzed in a denaturing 1.5% agarose-gel. Bands were visualized by EtBr-staining and UV illumination.

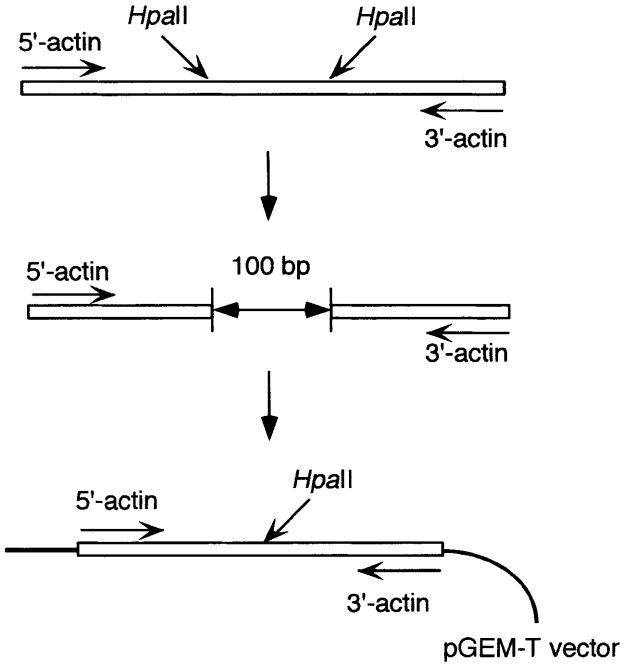

Fig. 4. Cloning of the internal standard plasmid for the competitive PCR analysis of cytoplasmic β-actin mRNA. For the construction of an internal standard applicable in primer competition, a new vector was constructed that contains the same primer-binding sites as the native cytoplasmic β-actin cDNA, but generates a 100-bp smaller fragment than the original PCR product. Since the size of this β-actin control plasmid is 3,305 bp (300-bp insert + 3,005-bp vector), 1 μg of this plasmid represents about 2.8×10^{11} copies of this molecule.

concentration of the internal control plasmid was chosen in a serial dilution ranging between 2.5×10^9 and 2.5×10^7 copies per reaction. Figure 5A shows the result of this competitive PCR, where the amount of β-actin mRNA in normal cells was estimated to be about 2.5×10^8 copies per reaction. In contrast, Wa cells induced to apoptosis were expected to contain fewer β-actin mRNA transcripts, which was also actually confirmed by competitive PCR (Fig. 5B), where the copy number of cytoplasmic β-actin in apoptotic Wa cells was estimated to be about 5×10^5copies per reaction. Thus, using the current method, the β-actin mRNA from apoptotic cells was determined to be about 1/500 of that of normal Wa cells. It is therefore evident that the induction of apoptosis actually results in a decrease of intact mRNA. To further investigate to what extent the HLA-DR β-chain mRNA was subjected to this degradation process, the amount of HLA-DRB mRNA in normal intact or apoptotic Wa cells was compared to each other by another round of competitive PCR. Firstly, the amount of intact mRNA between the two samples was adjusted so that equal copy numbers of cytoplasmic β-actin would exist in one reaction. For this the cDNA from normal Wa cells was diluted to the level of oxidant-treated Wa cells and the equimolarity of the diluted cDNA from normal Wa cells and the original cDNA solution from oxidant-treated cells were confirmed in an additional PCR using β-actin-specific primers (data not shown). Determination of the number of HLA-DRB1*0405 mRNA in normal and apoptotic Wa cells was then performed in the same manner as for the relative quantitation of β-actin mRNA, that is, using an internal control plasmid (Fig. 6) as a primer competitor and analyzing the PCR amplikons in TAE-agarose gels for the differential production of natural cDNA or internal standard signals (Fig. 7). Densitometric analysis revealed that the amount of HLA-DRB mRNA was similar in intact and apoptotic cells after normalization of their β-actin signals. Thus, the mRNA encoding MHC class II molecules and mRNA for β-actin undergo degradation by a similar, if not identical, mechanism, which seems to be induced by the apoptotic machinery.

Discussion

The process of programmed cell death in apoptosis is a cellular decay mechanism by which apoptosis-induced cells disintegrate after a prescribed protocol into small apoptotic bodies (vesicles), which are finally cleaned up by neighboring phagocytic cells. A large number of reports have been presented on the cellular events following commitment to apoptosis. These studies showed that the dying cell obviously does not abruptly cease to exert any cellular activity, but that apoptosis is rather accompanied by a vivid change in gene expression (Renno *et al.*, 1998; Wang *et al.*, 1997) as well as by the activation of some large signal cascades and the initiation of specific enzyme activities (Enari *et al.*, 1998). In the present study, it was questioned to what extent these apoptotic signals modify the expression of membrane proteins, especially in the case of human MHC class II molecules. The induction of apoptosis *in vitro* can be performed by a large variety of methods. As for EBV-transformed B cells, oxidant-induced cell death

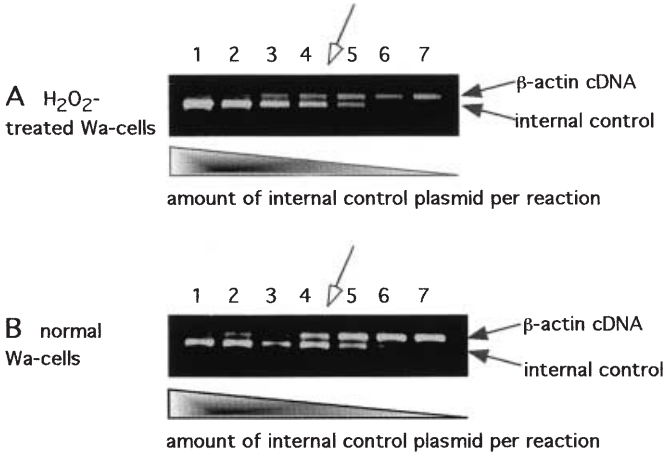

Fig. 5. Determination of cytoplasmic β-actin mRNA expression by competitive RT-PCR. Using the same amount of whole RNA for one reaction, cDNA was reverse-transcribed, and the relative amount of cytoplasmic β-actin was determined by semiquantitative RT-PCR. For primer competition, internal standards were included in the PCR with differing concentrations. The copy numbers of the control plasmids for each reactions are as following. **A.** Competitive PCR using cDNA from H_2O_2-treated Wa cells. Lane 1, 2.5×10^9 copies; lane 2, 1×10^9 copies; lane 3, 5×10^8 copies; land 4, 2.5×10^8 copies; lane 5, 1×10^8 copies; lane 6, 5×10^7 copies; lane 7, 2.5×10^7 copies. **B.** Competitive PCR using cDNA from normal Wa cells. Lane 1, 5×10^6 copies; lane 2, 2.5×10^6 copies; lane 3, 1×10^6 copies; lane 4, 5×10^5 copies; lane 5, 2.5×10^5 copies; lane 6, 1×10^5 copies; lane 7, 5×10^4 copies.

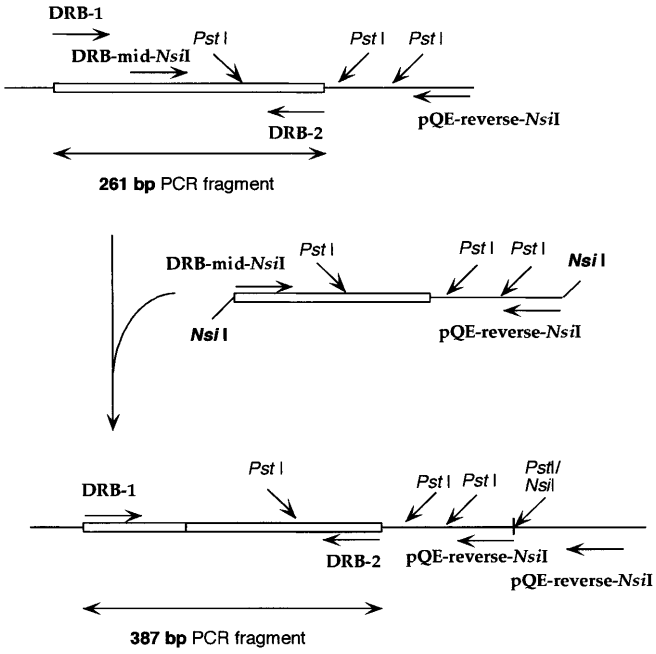

Fig. 6. Construction of an internal standard plasmid for the competitive PCR analysis of the HLA-DR β-chain mRNA expression. To compare the relative amount of HLA-DR mRNA expression, an internal control vector for the primer competition with HLA-DRB1*0405 mRNA was constructed. Since the size of the constructed HLA-DRB1*0405 internal standard is 3,835 bp, 1 μg of this plasmid represents about 2.4×10^{11} copies of this plasmid.

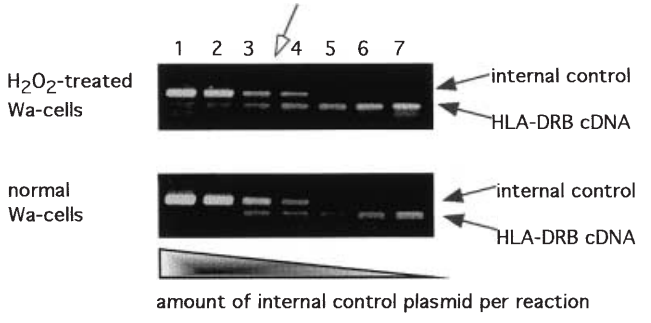

Fig. 7. Competitive RT-PCR of HLA-DR β-chain mRNA in normal and apoptotic Wa cells. The relative amount of intact cDNA was first adjusted between normal and apoptotic Wa cells on the basis of the results of the competitive PCR from the β-actin signal (see Fig. 5). Competitive PCR was then performed using equimolar amounts of cDNA with regard to β-actin copy numbers, using HLA-DR-specific primers. Lane 1, 5×10^6 copies; lane 2, 2.5×10^6 copies; lane 3, 1×10^6 copies; lane 4, 5×10^5 copies; lane 5, 2.5×10^5 copies; lane 6, 1×10^5 copies; lane 7, 5×10^4 copies.

has been known to be an efficient way to drive these cells to apoptosis (Lee and Shacter, 1997). However, since different cell lines show differential resistance to H_2O_2 (Lee and Shacter, 1997), first, the oxidant sensitivity of the particular EBV-transformed cell line as used in this study was determined. As shown in Fig. 1, incubation for 24 h in the presence of 1 mM H_2O_2 was sufficient to induce apoptosis in the EBV-transformed Wa cells.

Cells in the late apoptotic phase are characterized by lower forward scatter and higher side scatter values than viable cells when analyzed by flow cytometry, reflecting the smaller size and different consistency of the nucleus and cytoplasm (Sgonc and Wick, 1994). Since small cells have a largely reduced cell surface area, which might result in a size-dependent decrease of cell surface antigen display, the apoptosis-induced Wa cells were analyzed firstly for possible changes in their general cell morphology. Figure 2 shows that the phenotypic differentiation of Wa cells in terms of forward scatter is not largely affected during the first 24 h after induction of apoptosis. Obviously, no drastic changes in the cell volume and size (forward scatter) happen during this time when compared to normal Wa cells. Therefore, any modulation in the expression of cell surface molecules during this period should not be assigned to physical changes of the cell structure such as cell shrinkage or cellular disintegration but rather to an active down regulatory event. In this regard, the down modulation of class II molecules during apoptosis, as seen by the decrease of HLA-DR expression as displayed in the vertical axis (Fig. 2) in the dot plots, is to be considered as a real active down regulation.

The loss of a specific cell surface molecule by apoptosis is not limited to the present observation. Apoptotic neutrophils (Dransfield *et al.*, 1994) and thymocytes (Kishimoto *et al.*, 1995) have been reported to modulate cell surface proteins, and as for myeloma cells, syndecan-1 but not HLA class I molecules have been shown to be down regulated by programmed cell death (Jourdan *et al.*, 1998). Nevertheless, so far, no information has been available for the regulation of MHC class II molecules in apoptosis. The expression of HLA-DR molecules has been repeatedly confirmed to lie under transcriptional control (Rohn *et al.*, 1996). Thus, the reduction of MHC class II expression in apoptotic Wa cells might also be due to a decreased or even aborted transcription of HLA-DR mRNA. However since HLA-DRB mRNA has been determined to have a rather stable half-life of 14–16 h (Navarrete-Santos *et al.*, 1997), it is evident that there must be an additional mechanism which is responsible for the rapid down regulation (Fig. 2) of cell surface MHC class II expression, i.e. by actively decreasing the amount of available intact DRB mRNA. The onset of a specific RNA degradation machinery would the most plausible explanation.

Analysis of whole RNA extracted from apoptotic Wa cells shows that there is indeed a slight degradation, when compared to the RNA from normal cells (Fig. 3). To investigate, whether this degradation was

selectively biased to transcripts of the HLA-DR gene products, the mRNA signal of the HLA-DRB was compared to that of the cytoplasmic β-actin. The transcription products of this housekeeping gene have been used as a classical control for standardization of the amount of intact mRNA (Park *et al*., 1993). For a more accurate comparison of the respective mRNA, internal control plasmids were constructed (Fig. 4) and the amount of mRNA was semiquantitatively determined by competitive RT-PCR (Piatak *et al*., 1993). As shown in Fig. 5A, apoptotic Wa cells contained about 500 times less cytoplasmic β-actin mRNA than normal Wa cells, which indicated that RNA is rapidly degraded in these cells. After adjustment of the number of intact β-actin mRNA molecules, competitive RT-PCR was performed for again with cDNA from apoptotic and normal Wa cells using HLA-DRB-specific primers. Figure 7 shows that there is no apparent difference in the amount of HLA-DRB mRNA between normal and apoptotic cells when the relative amounts of intact mRNA were adjusted to each other using β-actin mRNA signal as a standard. That is, the ratio of intact DR β-chain mRNA to β-actin mRNA remained the same for apoptotic cells as for normal Wa cells, indicating that the decrease of HLA-DRB mRNA is not because of a specific DR β-chain targeted degradation but is due to general RNA degradation in these apoptotic cells. In conclusion, the cause for the rapid down-modulation of MHC class II molecules is mainly to be addressed to a decrease of intact DR mRNA molecules, which in turn is induced by an overall degradation of cytosolic RNA. Beyond the generally accepted view that dying cells might stop all metabolic activity and that they are virtually frozen in their current phenotypic state, in the present study, it was shown that there is an active modulation of protein expression, especially for cell surface proteins such as the class II molecules.

Despite these observations, reduction and elimination of mRNA molecules alone will not be sufficient to explain the whole mechanism behind the rapid loss of MHC class II molecules in apoptosis. It is therefore thinkable that there also exists a simple proteolytic degradation process (Kishimoto *et al*., 1995) which involves the destruction of intracellular components involved in the assembly and cell surface transport of new MHC class II molecules. On the other hand, it is also possible that there might be an additional mechanism at work, which gets rid of cell surface displayed MHC class II molecules by membrane-shedding as has been described for apoptotic neutrophils in downregulation of their CD16 molecules (Dransfield *et al*., 1994). In this process, the downregulation of surface CD16 correlates with a simultaneous increase of soluble CD16 concentration in serum, which might act there as a potent immunomodulator (Huizinga *et al*., 1990). Since the existence of soluble serum MHC class II molecules has been well proved, and since also alterations of soluble HLA-DR (sHLA-DR) concentrations upon immune responses have been observed, it can be postulated that these sHLA-DR molecules have also been derived from apoptotic immune cells. Some data supporting this point of view have been reported by several other groups, where an increased amount of sHLA-DR was detectable in HIV infected patients and after liver transplantation (Filaci *et al*., 1995) as well as in patients with multiple sclerosis (Ott *et al*., 1998), where massive apoptotic events are present (Ichikawa *et al*., 1996).

Therefore, if membrane sheddings of HLA-DR molecules indeed exist, the biological meaning of class II down regulation might also be understood in the same manner as for the loss of CD16 molecules in apoptotic neutrophils, which simultaneously ensures the prevention of apoptotic cells to participate in further immune reactions, and generates soluble surface receptors which might be involved in some other immunoregulatory functions. The possibilities mentioned so far have to be checked out in further studies. However, the results from the present study together with observations from some other groups reconfirm the presence of a specific mechanism selectively regulating not only membrane lipid and sugar composition but also the expression of cell surface proteins during apoptosis.

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References

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Dransfield, I., Buckle, A. M., Savill, J. S., McDowall, A., Haslett, C., and Hogg, N. (1994) Neutrophil apoptosis is associated with a reduction in CD16 (FcγRIII) expression. *J. Immunol.* **153**, 1254–1263.

Dumoulin, F. L., Bach, A., Leifeld, L., El-Bakri, M., Fischer, H. -P., Sauerbruch, T., and Spengler, U. (1997) Semiquantitative analysis of intrahepatic cytokine mRNAs in chronic hepatitis C. *J. Infect. Dis.* **175**, 681–685.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391**, 43–50.

Filaci, G., Contini, P., Brenci, S., Lanza, L., Scudeletti, M., Indiveri, F., and Puppo, F. (1995) Increased serum concentration of soluble HLA-DR antigens in HIV infection and following tranplantation. *Tissue Antigens* **46**, 117–123.

Gansbacher, B. and Zier, K. S. (1988) Regulation of HLA-DR, DP, and DQ expression in activated T cells. *Cell. Immunol.* **117**, 22–34.

Glimcher, L. H. and Kara, C. J. (1992) Sequences and factors: a guide to MHC class-II transcription. *Annu. Rev. Immunol.* **10**, 13–49.

Homburg, C. H. E., De Haas, M., von dem Borne, A. E. G. Kr., Verhoeven, A. J., Reutelingsperger, C. P. M., and Roos, D. (1995) Human neutrophils lose surface FcγRIII and acquire annexin V binding sites during apoptosis *in vitro*. *Blood* **85**, 532–540.

Huizinga, T. W. J., de Haas, M., Kleijer, M., Nuijens, J. H., Roos, D., and von dem Borne, A. E. G. Kr. (1990) Soluble FcgRIII in human plasma originates from release by neutrophils. *J. Clin. Invest*. **86**, 416–123.

Ichikawa, H., Ota, K., and Iwata, M. (1996) Increased Fas antigen on T cells in multiple sclerosis. *J. Neuroimmunol*. **71**, 125–129.

Jourdan, M., Ferlin, M., Legouffe, E., Horvathova, M., Liautard, J., Rossi, J. F., Wijdenes, J., Brochier, J., and Klein, B. (1998) The myeloma cell antigen syndecan-1 is lost by apoptotic myeloma cells. *Br. J. Haematol*. **100**, 637–646.

Katagiri, M., Ikeda, H., Maruyama, J., Wakisaka, A., Kimura, S., Aizawa, M., and Itakura, K. (1979) Evidence for two B-cell alloantigen loci in the HLA-D region. *Immunogenetics* **9**, 335–351.

Khan, M. A., Latif, N., Petrobelli, P., Yacoub, M. H., and Dunn, M. J. (1996) Construction of an internal standard for semi-quantitative polymerase chain reaction analysis of heat shock proteins. *Electrophoresis* **17**, 40–43.

Kishimoto, H., Surh, C. D., and Sprent, J. (1995) Upregulation of surface markers on dying thymocytes. *J. Exp. Med*. **181**, 649–655.

Konig, A., Happle, R., and Hoffmann, R. (1997) IFN-γ-induced HLA-DR but not ICAM-1 expression on cultured dermal papilla cells is downregulated by TNF-α. *Arch. Dermatol. Res*. **289**, 466–470.

Koppelman, B., Neefjes, J. J., De Vries, J. E., and De Waal, M. R. (1997) Interleukin-10 down-regulates MHC class II αβ/peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling. *Immunity* **7**, 861–871.

Lee, Y. J. and Shacter, E. (1997) Bcl-2 does not protect Burkitt's lymphoma cells from oxidant-induced cell death. *Blood* **89**, 4480–4492.

Maccarrone, M., Nieuwenhuizen, W. E., Dullens, H. F., Catani, M. V., Melino, G., Veldink, G. A., Vliegenthart, J. F., and Finazzo Agro, A. (1996) Membrane modifications in human erythroleukemia K562 cells during induction of programmed cell death by transforming growth factor beta 1 or cisplatin. *Eur. J. Biochem*. **241**, 297–302.

Martin, S. J., Reutelingsperger, C. P. M., McGahon, A. J., Rader, J. A., van Schie, R. C. A. A., LaFace, D. M., and Green, D. R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of bcl-2 and abl. *J. Exp. Med*. **182**, 1545–1556.

Maudsley, D. J. and Pound, J. D. (1991) Modulation of MHC antigen expression by viruses and oncogenes. *Immunol. Today* **12**, 429–431.

Miller, J. and Sant, A. J. (1995) Introduction: MHC class II-restricted antigen presentation. *Seminars Immunol*. **7**, 343–346.

Navarrete-Santos, A., Rieman, D., Thiele, K., Kehlen, A., Navarrete-Santos, A., and Langner, J. (1997) Constitutive expression of HLA class II mRNA in synovial fibroblast-like cells from patients with rheumatoid arthritis. *Immunol. Lett*. **58**, 53–58.

Ott, M., Seidl, C., Westhoff, U., Stecker, K., Seifried, E., Fischer, P. A., and Grosse-Wilde, H. (1998) Soluble HLA class I and class II antigens in patients with multiple sclerosis. *Tissue Antigens* **51**, 301–304.

Otten, L. A., Steimle, V., Bontron, S., and Mach, B. (1998) Quantitative control of MHC class II expression by the transactivator CIITA. *Eur. J. Immunol*. **28**, 473–478.

Park, J. H., Mitnacht, R., Torres-Nagel, N., and Hunig, T. (1993) T cell receptor ligation induces Interleukin (IL) 2R β-chain expression in rat CD4, 8 double positive thymocytes, initiating an IL-2-dependent differentiation pathway of $CD8\alpha^{+}/\beta^{-}$ T cells. *J. Exp. Med*. **177**, 541–546.

Park, J. H., Choi, E. A., Cho, E. W., Hahm, K. S., and Kim, K. L. (1998a) Maltose binding protein (MBP) fusion proteins with low or no affinity to amylose resins can be single-step purified using a novel anti-MBP monoclonal antibody. *Mol. Cells* **8**, 709–716.

Park, J. H., Cho, E. W., Lee, Y. J., Chung, J., Hahm, K. S., and Kim, K. L. (1998b) Generation of anti-HLA-DR4 specific antibodies by immunization of recombinantly expressed allelic subtype specific region of the HLA-DRB1*0405 molecules. *J. Biochem. Mol. Biol*. **31**, 111–116.

Piatak, M. Jr., Luk, K. -C., Williams, B., and Lifson, J. D. (1993) Quantitative competitive polymerase chain reaction for accurate quantiation of HIV DNA and RNA species. *BioTechniques* **14**, 70–79.

Renno, T., Hahne, M., Tshopp, J., and MacDonald, H. R. (1996) Peripheral T-cells undergoing superantigen-induced apoptosis *in vivo* express B220 and upregulates Fas and Fas ligand. *J. Exp. Med*. **183**, 431–437.

Renno, T., Attinger, A., Rimoldi, D., Hahne, M., Tschopp, J., and MacDonald, H. R. (1998) Expression of B220 on activated T cell blasts precedes apoptosis. *Eur. J. Immunol*. **28**, 540–547.

Rohn, W. M., Lee, Y. J., and Benveniste, E. N. (1996) Regulation of class II MHC expression. *Crit. Rev. Immunol*. **16**, 311–330.

Schackelford, D. A., Lampson, L. A., and Strominger, J. L. (1981) Analysis of HLA-DR antigens by using monoclonal antibodies: recognition of conformational differences in biosynthetic intermediates. *J. Immunol*. **127**, 1403–1410.

Sgonc, R. and Wick, G. (1994) Methods for the detection of apoptosis. *Int. Arch. Allergy. Immunol*. **105**, 327–332.

Villa, P., Kaufman, S. H., and Earnshaw, W. C. (1997) Caspases and caspase inhibitors. *Trends Biochem. Sci*. **22**, 388–393.

Walker, P. R., Kokileva, L., LeBlanc, J., and Sikorska, M. (1993) Detection of the initial stages of DNA fragmentation in apoptosis. *BioTechniques* **15**, 1032–1040.

Wang, S., DiBenedetto, A. J., and Pittman, R. N. (1997) Genes induced in programmed cell death of neuronal PC12 cells and developing sympathetic neurons *in vivo*. *Dev. Biol*. **188**, 322–336.

Zier, K., Gansbacher, B., Ikegaki, N., Kennett, R., and Polakova, K. (1989) Expression of HLA-DR mRNA in T cells following activation is early and can precede DNA synthesis. *Autoimmunity* **5**, 59–70.