Reumatismo, 2004; 56(3):156-161

LAVORO ORIGINALE

L'Apoptosis e la necrosi aumentano l'antigenicità di proteine riconosciute dagli anticorpi antinucleari

Apoptosis and necrosis increase antigenicity of proteins recognized by antinuclear antibodies

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RIASSUNTO

Obiettivo. *Lo scopo di questo studio è quello di indagare se l'apoptosis e la necrosi aumentano l'antigenicità delle proteine riconosciute da anticorpi antinucleo.*
Materiale e metodi. *Cellule HEp-2 sono state coltivate in condizioni standard; l'apoptosis è stata indotta con camptecina e la necrosi con cloruro di mercurio. L'antigenicità delle proteine estratte dalle cellule è stato testata su membrane di nitrocellulosa e sondata con sieri positivi o negativi per anticorpi antinucleo utilizzando un sistema ELISA a luminescenza (luminescent).*
Risultati. *Le alterazioni apoptotiche nelle cellule HEp-2 sono apparse entro 24 ore dall'esposizione alla camptoicina, mentre i segni di necrosi si sono evidenziati più precocemente. La luminescenza si è dimostrata significativamente superiore nei sieri ANA positivi che nei controlli ANA negativi. Gli antcorpi antinucleari sieirici riconoscono meglio gli antigeni da cellule apoptotiche e necrotiche rispetto ai controlli che non hanno subito trattamenti chimici.*
Conclusioni. *L'apoptosi e la necrosi incrementano la capacità legante degli ANA attraverso una migliore disponibilità di antigeni intracellulari o svelando epitopi criptici.*

Reumatismo, 2004; 56(3):156-161

INTRODUCTION

Autoimmune diseases are characterized by the presence of auto-antibodies against a wide variety of intracellular auto-antigens; in normal conditions intracellular proteins do not raise the cell surface, nevertheless under apoptosis or necrosis the intracellular proteins are translocated to cell membranes and extra cellular space, therefore the molecules suffer conformational changes that play a role in the antigen driven response (1).
Apoptosis is a physiologic mechanism that preserves the tissue development and protects organisms by removing defective and senescent cells, the clearance of apoptotic material avoid the "toxic accumulation" (2). Defects in apoptosis could shut down the tolerance in genetically predisposed individuals.
A failure of Fas/FasL pathway could allow the survival of autoimmune lymphocytes which escape the negative selection into the thymus. Fas defects have been demonstrated in animal models and human SLE. In addition, mutations of C3, C1q, Fc receptors and pentraxines result in a defective recognition and a lack of cleaning of cellular debris which trigger off autoantibodies (3-9).
In clinical practice is difficult to define whether autoantibody response is triggered by native or cryptic antigens produced under apoptosis or necrosis. To answer this important question, apoptosis or necrosis was chemically induced in HEp-2 cells and the protein antigenicity was probed with antinuclear antibodies by a luminescent-spot-ELISA (LsELISA) test.

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MATERIAL AND METHODS

Sera. One hundred and sixty two sera were studied. Eighty one had positive ANA on HEp-2 cells (titer higher than 1:80). Sera were obtained from patients with systemic lupus erythematosus (SLE) n=60, primary Sjögren syndrome (SS) n=5, progressive systemic sclerosis (SE) n=15 and mixed connective tissue disease (MCTD) n=1 (10-13). The remaining eighty one sera were obtained from negative ANA healthy controls.

Cell cultures. Epithelial HEp-2 cells obtained from ATTC were grown in DMEM medium supplemented with 10% fetal calf serum (Gibco, BRL. Gaithersburg MD). Cells were suspended and plated in culture flasks at a density of $5x10^6$/ml and incubated at 37°C 5% CO_2 and 85% of relative humidity. Cells in logarithmic growth phase ($1X10^6$) were incubated 24 h with 20 mM camptothecin (Sigma. St Louis, MO) (14). Another culture was incubated 24 hours with 5 mM mercuric chloride to induce necrosis (15). Control cells were cultured without chemicals. Changes in cell morphology were determined by light microscopy. Apoptotic fragments were detected by TUNEL (Roche Diagnostics GmbH, Mannheim, Germany).

Indirect Immunofluorescence. Antinuclear antibodies were tested on HEp-2 cells, the slides were incubated 30 minutes with serum diluted 1:80 to 1:10,280, followed by PBS washings and 30' incubation with FITC-labeled rabbit anti-human polyclonal gamma globulin (IgG, IgA and IgM, Sigma. St Louis, MO), after washings the slides were evaluated in epifluorescent microscopy.

Antigen extraction. Cells were harvested with a scraper and spun at 1200 rpm/10 minutes, then suspended in denaturing lysis buffer (1% SDS, 0.25M EDTA, 2mM bis-mercaptoethanol, 1mM tris pH 7.5, 5mM PMSF and adjusted to 1 ml H_2O). The soluble antigens were extracted by sonication, lysates were centrifuged at 12000 rpm/10 minutes and supernatants used for analysis. Protein concentration was measured by spectrophotometer at 280 nm.

Luminescent-spot-ELISA (LsELISA). Cell extracts adjusted to 2 μg/μl were immobilized onto nitrocellulose membranes (BioBlot-NC, Cambridge, MA) and dried at room temperature, then blocked with 3% non-fat milk dissolved in PBS. Antigenic spots were incubated 30 minutes with a 1:3000 dilution of serum, membranes were washed in PBS-0.1% Tween 20 and incubated 30' with a 1:1000 dilution of goat anti-human polyvalent gamma globulin (IgG, IgA and IgM) labeled with peroxidase, after washings the luminescent reaction was developed with ECL (Amersham Pharmacia Biotech. Piscataway, NJ). Membranes were analyzed in a Chemi-BioRad image analyzer. The protein concentration of cell extracts was decided by an optimization curve taking into account sensitivity and specificity.

Preparation of F(ab')2 fragments. Sera were digested as follows: One hundred ml of serum were incubated during 22 h at 37°C with 200 ml of pepsin (Sigma. St Louis, MO), the enzyme was activated by 0.1M acetate buffer, pH 3.6 (1 mg/ml). After incubation the tubes were spun 20' at 2500 rpm and the supernatant was saved and neutralized with 0.15M Tris-HCl, pH 8.2 (16). F(ab')2 fragments were tested by LsELISA as previously described.

Statistics. Mean values obtained by the image analyzer were submitted to a multivariate analysis, the R value of intra and inter-assay was determined by canonical correlation. The critical comparisons between positive and negative ANA sera using normal, apoptotic and necrotic cell extracts was calculated by paired t-Test using the NCSS program.

RESULTS

Apoptosis and necrosis. Cellular changes were produced by the chemicals within 24 hours. The apoptotic cells showed: blebs, apoptotic bodies and chromatin fragmentation, therefore the ~98% of cells incubated with camptothecin exhibited positive TUNEL, meanwhile the percent of cells incubated without it was <1%. The mercuric chloride induced necrotic changes in the ~99% of cells within 12 hours incubation (Figure 1 and 2).

Recognition of HEp-2 cell antigens. Positive ANA serum recognized the antigens from normal control cells; in sharp contrast the ANA negative controls did not recognize the HEp-2 cell antigens ($p < 0.0001$). Table I and figure 3. Results of this assay were reproducible; therefore the intra-assay and inter-assay correlation showed a significant R value (1.00), in five replicas.

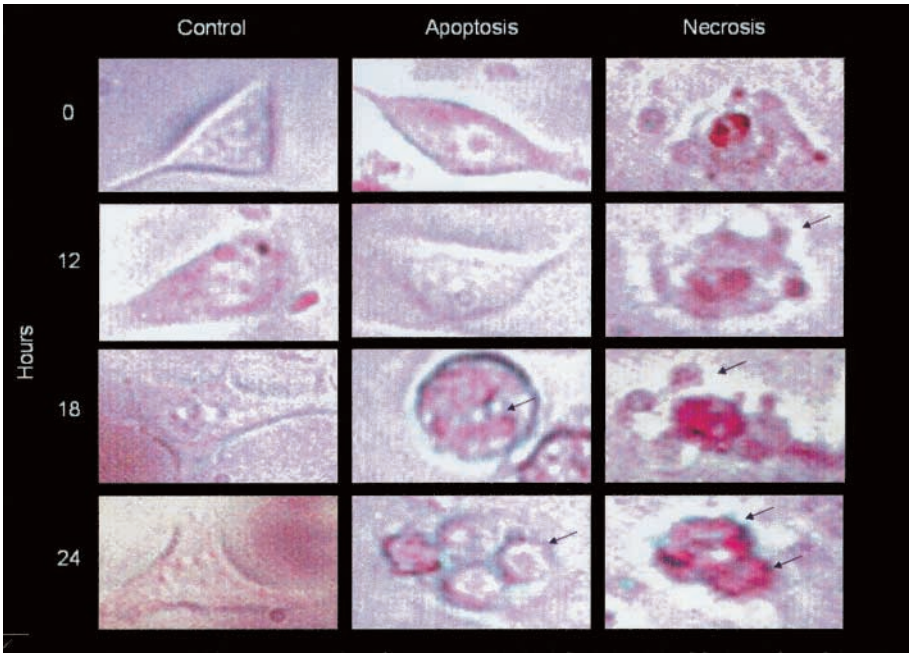

Figure 1 - A representative panel of HEp-2 cells cultured and evaluated under light microscopy. Living cells (left); apoptotic cells (middle) showing blebs and apoptotic bodies (arrows); necrotic cells (right) showing cell disruption.

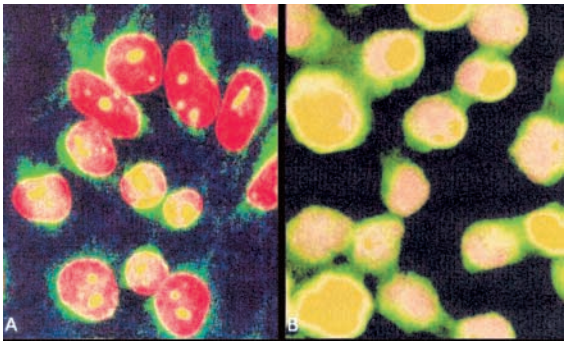

Figure 2 - TUNEL in HEp-2 cells. A Control cells negative for TUNEL (green), the nuclei of living cells are rather stained in red by the 0.2% propidium iodide. B. Cells incubated 24 hours with camptothecin are tagged in green by the TUNEL reagent, all the cells are death.

Table I - Statistical data of LsELISA units in 162 sera (n=81 +ANA, 81 -ANA.

	Sera/Antigen	*Mean/sd*	*Variable*	*P value*
A	+ANA/Living cells	753±31.8	A vs. C, E	< 0.0001
B	-ANA/Living cells	1.04±0.70	A vs. B	< 0.0001
C	+ANA/Apoptosis	1684±93	C vs. E	0.2624
D	-ANA/Apoptosis	1.04 ± 0.59	C vs. D	< 0.0001
E	+ANA/Necrosis	1532±98	E vs. F	< 0.0001
F	-ANA/Necrosis	1.04± 0.46	F vs. D, B	1.0000

P < 0.05 means significant different by paired t Test

Apoptosis and necrosis enhance antigenicity. The ANA reactivity was significantly higher using apoptotic and necrotic cell extracts than those values obtained with the control cell extracts ($p < 0.0001$). In addition, no differences were found in positive ANA sera using apoptotic and necrotic cell extracts (Table I, Figure 3).

HEp-2 cell antigens recognition by F(ab')$_2$. To rule out the non-specific binding of the serum to cell antigens the following experiments were carried out:

1) The influence of the complement was ruled out pre-heating the serum at 56°C for 30 minutes; under this approach the luminescent values did not show significant variations.
2) To assess the influence of self Fc interaction, the sera were digested with pepsin to obtain F(ab')$_2$ fragments. The LsELISA values of F(ab')$_2$ fragments were similar to those of total serum in presence of anti-F(ab')$_2$ as second antibody; nevertheless they were negative with anti-Fc as second antibody (Table II).
3) To demonstrate the antigenic specificity, the membranes were coated with 3% bovine serum albumin, and the positive and negative ANA sera were tested; under this condition sera did not reacted.

Not differences of ANA reactivity between subgroups of patients (SLE, SE, SS, and MCTD) were

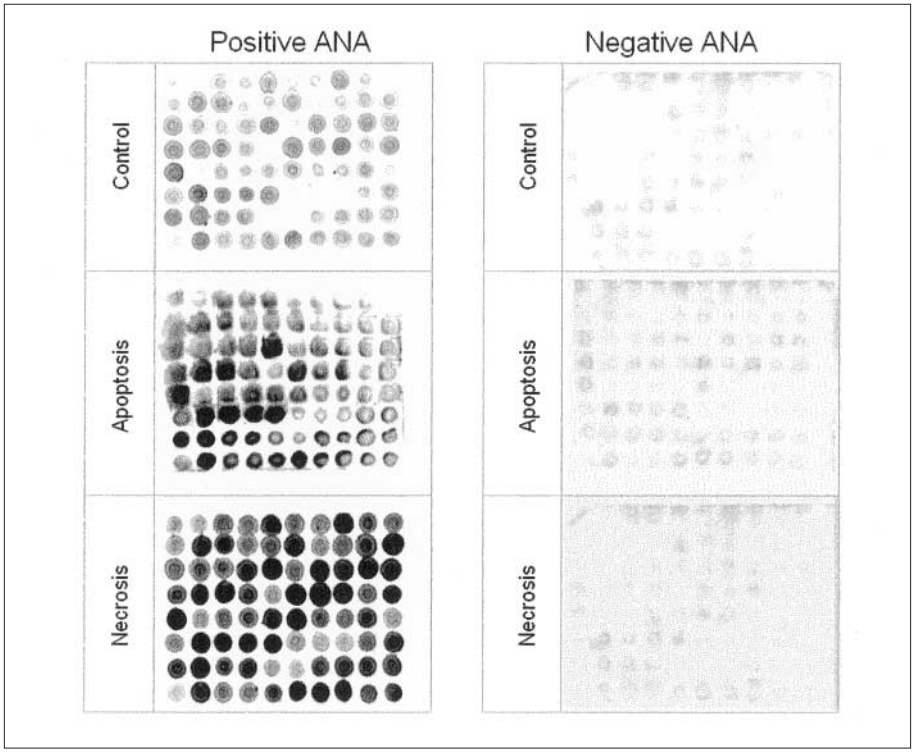

Figure 3 - LsELISA. The panels show the ANA reactivity images of the control, apoptotic and necrotic cell extracts probed with negative and positive ANA sera.

Table II - Statistical data of LsELISA units in 81 +ANA sera heated or digested.

	Sera/Antigen	*Mean/sd*	*Variable*	*P value*
A	ANA sera/Control cells	753±31.8	A vs. B	0.5023
B	ANA sera/56°C/Control cells	722±32.1	B vs. C	0.4676
C	ANA $F(ab')_2$/Control cells/Anti-$F(ab')_2$	740±32.1	A vs. C	0.4239
D	ANA $F(ab')_2$/Control cells/Anti-Fc	0.006±0.002	D vs C	P<0.0001
E	ANA sera/Apototic cells	1684±93	E vs. F	0.9845
F	ANA sera/56°C/Apoptotic cells	1682±92	F vs. G	0.8778
G	ANA $F(ab')_2$/Apoptotic cells/Anti-$F(ab')_2$	1702±90	G vs. E	0.8939
H	ANA $F(ab')_2$/Apoptotic cells/Anti-Fc	0.001±0.009	H vs. G	P<0.0001
I	ANA sera/Necrotic cells	1532±98	I vs. J	0.9936
J	ANA sera/56°C/Necrotic cells	1533±97	J vs. K	0.9869
K	ANA $F(ab')_2$/Necrotic cells/Anti-$F(ab')_2$	1536±97	K vs. I	0.9806
L	ANA $F(ab')_2$/Necrotic cells/Anti-Fc	0.004±0.003	K vs L	P<0.0001

$P < 0.05$ means significant different by paired t Test

observed, using normal, apoptotic or necrotic cell extracts.

DISCUSSION

In this study we showed that the antigenic properties of molecules recognized by antinuclear antibodies was appraised using apoptotic or necrotic cell extracts, the main results were the following:

1) Positive ANA sera specifically recognize autoantigens from HEp-2 cells by LsELISA.
2) Normal human sera did not recognize the HEp-2 cell antigens.
3) The ANA binding to cell extracts was increased in antigens produced by apoptosis or necrosis.

Apoptosis is a physiologic condition triggered by internal or external conditions; after apoptosis the

cellular material is removed by a number of conserved mechanisms that involve the recognition, phagocytosis and degradation of the cellular remains. A failure in the apoptotic material clearance would produce toxic accumulation, which stimulate an antigen driven response (17). Autoantigens are cleaved by apoptosis and/or necrosis, and then intracellular molecules raise the cell membrane or the extra-cellular space becoming accessible to the antigen presenting cells (18-22).

Pioneer observations demonstrated that treatments with procainamide, hydralazine or other drugs may lead to ANA production, this suggested that autoimmunity would be chemically induced (23). Chemotherapy in cancer induces autoantibodies; for example the camptothecin increases the DNA topoisomerase I levels leading anti-nucleolar or anti-La autoantibodies (24, 25). In addition the intoxication with heavy metals as the mercuric chloride produces anti-nucleolar antibodies (26). In summary, the tolerance would be braked down by chemicals and others factors; however in clinical practice it is difficult to determine who drives the autoantibody production.

With the notion that autoantibodies are hallmarks and fingerprints of autoimmunity (27), ANA sera were used to explore whether apoptosis or necrosis were involved in the antigen driven response in autoimmune patients. Present results suggest that cryptic antigens produced under experimental apoptosis or necrosis react better to ANA than the native antigens; this result is coincident with others who had shown that apoptosis increases the HEp-2 cell antigenicity (28).

The apoptosis induces antigen redistribution on cell membrane, by this process the intracellular molecules are exposed by the membrane polarity shift; therefore the loss of phospholipids asymmetry allows the interaction between the membrane phospholipids with chromatin, ribonucleoproteins and other intracellular molecules via phosphatidylserine (29, 30). In addition, the protein cleavage would release neo-antigens which potentially increase the ANA reactivity (31, 32). By these results we would like to suggest that the apoptosis and necrosis are involved in the antigen driven response of antinuclear antibodies.

Acknowledgements. Supported by CONACYT grant number 38054N (R. Herrera-Esparza).

SUMMARY

Objective. Present study addresses the issue whether apoptosis and necrosis increases the antigenicity of proteins recognized by antinuclear antibodies.

Material and methods. HEp-2 cells were cultured in standard conditions; apoptosis was induced by camptothecin and necrosis by mercuric chloride. Protein antigenicity of cell extracts was tested onto nitrocellulose membranes and probed with positive or negative sera for antinuclear antibodies by a luminescent-dot-ELISA system.

Results. Apoptotic changes in HEp-2 cells appeared by 24 hours of camptothecin exposure, meanwhile the necrotic features become visible earlier. Luminescence was significantly superior in ANA positive sera than in ANA negative controls. Antinuclear antibody sera recognized better the antigens from the apoptotic and necrotic cells than controls without chemical treatments.

Conclusions. Apoptosis and necrosis increase the ANA binding by better availability of intracellular antigens, or by disclosing cryptic epitopes.

Key words - Apoptosis, necrosis, antinuclear antibodies, antigen cleavage.

Parole chiave *- Apoptosis, necrosi, anticorpi antinucleari Scissione di antigene.*

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