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Cytoskeleton actin changes in IL-2 activated cells

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SUMMARY

In the present study we analysed the changes in cytoskeletal actin in lymphoid cells following IL-2 activation and during cell interactions by means of light and electron microscopy, immunofluorescence and molecular analysis. By morphological analysis we observed a higher fluorescence in the activated cells than in the quiescent ones with no modifications in the cytoskeleton pattern comparing activated to resting cells. The results of molecular analysis indicate that, after IL-2 activation, there is a reorganisation of the actin component of the cell cytoskeleton accompanied by the differential expression of the corresponding genes. A future study will be extended to the analysis of others components of the cytoskeleton network.

INTRODUCTION

Following cell activation, the transcription of many genes controlling proliferation, motility and new protein synthesis is triggered. Previously we have studied morphological changes in cells with LAK lynphokine activated killer) activity induced *in vitro* by interleukin-2 (Nano *et al.*, 1996). We identified LAK cells with CD4 positive phenotype and studied the association of this function with some enzymatic activities and cell cycle phases (Nano *et al.*, 1995), and with a large number of sequences corresponding to genes involved in cellular communication, signal transduction and cell locomotion (Damiani *et al.*, 1998). The aim of this preliminary study was to evidence actin changes in the lymphoid cell cytoskeleton induced by IL-2 activation. The role of the cytoskeleton in the modifications induced by activation signals seems not only to be of the structural type since it has been reported that the three main components of the cell cytoskeleton (actin: thin filaments, vimentin: intermediate filaments, and tubulin: microtubules) are involved in the signalling processes mediated by the cell membrane (Jordan *et al.*, 1998). We limited our study to cytoskeletal actin changes, but the work is in progress as regards the expression of the other main components of the cytoskeleton, in particular vimentin and tubulin gene expression.

MATERIALS AND METHODS

rIL-2 activated cells

Peripheral blood mononucleated cells (PBMC) from normal subjects were isolated by a 1077 den-

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sity gradient (Istopaque Gibco) and pulsed for 12 hours with phytohemagglutinin (PHA Gibco, 1% final concentration in culture medium). Non-adhering cells were then harvested and cultured in RPMI 1640 (Dutch modification) supplemented with foetal calf serum (10%) in the presence of recombinant interleukin-2 (rIL-2 Eurocetus, 100pg/ml) for eight days. The complete medium was renewed every two days.

LAK-target cell co-cultures

Cell from various human cell lines (Chang ATCC CL 13, K562 ATCC CCL 243, HeLa ATCC CL 2, fresh isolated tumoral cells at 1st passage in culture) were considered as targets. The target cells were grown on 24x24 mm slides and IL-2 treated lymphocytes (LAK) in a 1:2 ratio were added there.

Morphological study

For the study of cell morphology, May–Grunwald Giemsa staining was performed on samples of lymphocytes before and after IL-2 activation and on samples of LAK-target cells obtained at different times (2, 4, 12, 24 hrs) of co-cultivation.

Electron microscopy

The cells were fixed with 1.5% glutaraldehyde in 0.7M cacodylate buffer (pH 7.4) for 3 hours at 4°C, washed in 0.1 M cacodylate buffer with 7% sucrose and post-fixed with 1% OsO_4 in the same buffer for 2 hours at 4°C. After several washings, the cells were pre-embedded in 2% agar in buffer, dehydrated in graded ethanols and embedded in Epon 812. Thin sections were stained with saturated uranyl acetate in 50% acetone and with Reynold's lead citrate. The specimens were examined with a Zeiss transmission electron microscope EM 900 operating at 50 kV.

Immunofluorescence analysis

Coverslips with co-cultured cells were fixed in methanol (5 minutes at-20°C) and washed in PBS containing 2mM EGTA and 2mM $MgCl_2$ (buffer A, pH 7.4). The specimens were fixed in 3% paraformaldehyde in PBS containing 1mM $MgCl_2$ and 1mM$CaCl_2$ (buffer B pH 7.4 for 30 min at R.T.), permealized with Triton X-100 (0.2% in buffer B for 15 min at R.T.) then incubated in the presence of TRITC-conjugated phalloidin (1:50 for 45 min at RT in humidified chamber).

Nuclei were stained with Hoechst 33342 (5µg/ml for 10 min at R.T.).

The coverslips were mounted with 90% glycerol in PBS and observed in epifluorescence with a NIKON OPTIPHOT microscope.

All chemicals were obtained from Sigma Chem. Co. (St.Louis, MO).

Molecular analysis

mRNAs from IL-2 stimulated and unstimulated PBL were obtained by the guanidinium thyocyanate method (Trizol reagent, Gibco BRL) and retrotranscribed with the anchored primer CGGATCC-CT_{11}VN. Quantitative RT-PCR was performed using commercial primers annealing β-actin gene (Clontech) and primers annealing conserved domains of actin genes (upper: ACGTCGCCCTGGACTTCG-AG; lower: GCGGATGTCCACGTCACA). Amplification reactions were performed in a 10µl volume containing 10mM Tris-HCl pH 9.0, 500mM KCl, 3mM $MgCl_2$, 200µM of each dNTP, 200ng of each primer, 1-50ng of first strand cDNA and 0.5u of Taq DNA polymerase (Amersham Pharmacia Biotech). Reactions were assembled at 0°C and the tubes were quickly transferred to a Hybaid thermal cycler preheated at 94°C. The program used has a first step at 94°C for 3min followed by 40 cycles at 94°C for 30sec, 54°C for 1min and 72°C for 2min. The samples were finally incubated at 72°C for 10min, at 60°C for 10min. 5µl of the amplified DNA were separated by electrophoresis for 2 hours at 5 Volts/cm onto a 3% (w/v) agarose gel (1% SeaKem GTG-FMC; 2% NuSieve GTG-FMC) (FMC Corp., USA) with TAE buffer containing 0.5µg/ml of ethidium bromide (Sambrook *et al.* 1989); the patterns were visualized with a long-wave UV lamp device.

RESULTS AND DISCUSSION

The modifications in the cell surface in effector and target cells during their interaction were studied in the same samples under light and electron microscope. Figure 1 shows lymphoid cells interacting, respectively, with a cell of the K562 line (a) and with a cell of the Chang line (b), stained with May Grunwald-Giemsa. We observed structures, such as protruding arms spreading from the effector cells that contacted with the target. In the

area of close contact between the cells, a faint zone, probably due to modifications of the surface proteins, was evident. The same pattern was observed during the interactions with all the studied targets. Details of the adhesion structures are well evidenced at the ultrastructural level: figure 1d shows the finger-like cytoplasmic extroflessions of an activated lymphocyte.

The cytoskeleton actin proteins were studied with immunofluorescence in resting and activated lymphocytes and in co-cultures of activated lymphocytes with target cells. Comparing activated to resting cells, we observed a higher fluorescence in the activated cells than in the quiescent ones, with no evident modifications of the cytoskeleton pattern. In fact, after stimulation with IL-2, the fibers appeared organised along the polarized body of the cells that showed the typical shape induced by activation (hand mirror) without apparent modification of their structure (data not shown).

Considering the interacting cells in the effector-target co-cultures, evident modifications, in particular of actin fibers, were observed. In fact the actin filaments revealed important reorganization patterns with the appearance of intense fluorescent spots, probably an expression of cytoplasmic areas in cleavage phase, particularly localized between the two cells (Fig. 1c). A similar situation, involving the F-actin reorganization, was found in membrane blebbing during stress-induced apoptosis (Huot *et al.*, 1998; Bonanno *et al.*, 2000; Spano *et al.*, 2000).

In vertebrates, the actin proteins of the cytoskeleton microfilaments are responsible for cell stability and are involved in cell plasticity and locomotion (β and γ–actin) (Henikoff *et al.*, 1997; Erba *et al.*, 1986). The gene coding for β-actin is considered a housekeeping gene and is used in molecular

Fig. 1 - Light, fluorescence and electron microscopy of lymphokine activated cells (LAK) involved in cell-target interaction. Interaction between LAK cells and the K562 cell line (a) and LAK cells and the Chang cell line (b,c,d). In light micrographs (a, b) a faint zone in the area of close contact of the target cellwith structures protruding from the effector cell is evident. In actin immunofluorescence (c), an activated lymphocyte shows a high intensity, and numerous spots are distributed on the cell surface. In d, an ultrastructural detail of finger-like cytoplasmic extroflessions of a LAK cell is shown. The presence of some erythrocytes is due to the technical procedure to preserve the sample during the enrichment. (a, b: x1,200; c: x2,400; d: x5,000).

biology as a control in studies on gene activation and differentiation. On the other hand, some researchers (Spanakis *et al.*, 1993; Schena *et al.*, 1996) found that the transcribed gene is overexpressed following stress conditions and cell activation. Cytoskeletal actin seems not only o have a structural role, since the actin filaments interact with the Rho GTPase family involved in the control of trancription and adhesion (Hall *et al.*, 1998). The Rho proteins mediate the cytoskeleton reorganization at the interface between the outer and inner cell membrane, following precise environmental signals. As a consequence, the genes coding for cytoskeletal actins are under regulation, and their trancription should increase following stress signals. Our molecular data confirm this hypothesis. In fact, quantitative PCR using primers for the highly conserved regions that perfectly anneal all known genes of the actin family, revealed patterns compatible with the two different conditions: unstimulated and IL-2 stimulated cells. In figure 2 are compared the electrophoretic patterns of amplified cDNA fragments obtained starting from both quiescent and IL-2 stimulated lymphocytes using primers annealing human β actin (lanes 2,3) or conserved actin motives (lanes 4,5). No differences between unstimulated and stimulated cells were observed using the commercial primer for β-actin. On the contrary, using a primer designed for conserved regions of actin genes (see materials and methods), a differential pattern of the two conditions is evident: in particular, before activation, we observed an intense band of about 350bp and a very faint band of about 200bp. After IL-2 activation, we observed the opposite situation: the 350bp band showed a high reduction in intensity and the 200bp band appeared much more intense. This situation is compatible with several hypotheses: alternative splicing of the same actin genes or expression of new actin genes after cell activation.

To complete the study on cytoskeleton organisation, the modifications and rearrangement of vimentin and tubulin gene expression will be studied.

Fig. 2 - Electrophoretic pattern obtained with cDNA from quiescent (-) and IL-2 stimulated lymphocytes (+) amplified using commercial primers annealing human b-actin gene (lanes 2,3), or primers annealing conserved motives of actin gene family (lanes 4,5). In the first lane the is present the molecular weight marker pBR322/MspI.

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