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Changes in Actin Organization During the Cytotoxic Process

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Changes in organization of F-actin during the cytotoxic process between NK and K562 cells have been observed and studied using confocal laser scanning microscopy and quantitative fluorescence microscopy. An increase in F-actin content and orientation of F-actin towards the target cell have been observed in conjugated NK cells. The increase in F-actin content probably reflects activation of the NK cell for the killing process. An increase in F-actin content in the conjugated K562 cell, occurring simultaneously with the appearance of filamentous actin structures that often originated at the contact place with the NK cell, was also observed. These changes were delayed compared to the increase in F-actin content in the NK cell and were accompanied by increasing cytotoxic activity. This indicates that they were results of the interaction of the K562 cell with the activated NK cell. The possible role of target cell microfilaments in the cytotoxic process is addressed.

Key terms: Natural killer, target cell, F-actin, quantification, confocal microscopy

NK cells are usually defined by their ability to kill in vitro, without previous sensitization and/or MHC-restriction, a variety of tumor cells, virus-infected cells, and immature cells (17). It is generally accepted that close contact between NK and target cell (conjugate formation) is required for the killing process to take place. The exact mechanism by which an NK cell destroys its target cell has not been completely elucidated. The most widely accepted model assumes incorporation of pore-forming proteins, secreted by the NK cell, into the membrane of the target cell (5,7,21). However, other possible mechanisms have been suggested (4, for a review see 20).

The role of microfilaments in the cytotoxic process has been addressed by the observation that microfilament-destroying drugs can exhibit inhibitory effects on the cytotoxic activity (9,11). Conjugate formation and postlytic recycling of killer cells have been identified as a cytochalasin sensitive step (11), whereas ML-9, a selective inhibitor of myosin light chain kinase, inhibited the programming for lysis phase (9). Concentration of F-actin in the effector cell towards the contact zone with the target cell has been observed, using fluorescence microscopy and actin specific probes (2,3,15), again indicating the involvement of actin in the cytotoxic process.

Using confocal laser scanning microscopy and quantitative fluorescence microscopy, we have studied morphological and quantitative aspects of actin organization during the cytotoxic process, with special attention to F-actin of the target cells (K562). We have used fluorescently labelled phalloidins as F-actin specific probes (19). Phalloidins bind specifically and stoichiometrically to F-actin, enabling its quantification (8). For the quantification experiments we have chosen rhodamine phalloidin, which exhibits the greatest fluorescence enhancement of all fluorescent phalloidin derivatives upon binding to F-actin (8) (reducing therefore problems accompanied with eventual nonspecific staining). Confocal laser scanning microscopy experiments were performed using rhodamine phalloidin or BODIPY 581/591 phalloidin, the fluorescent derivatives of phalloidin that exhibit a low photobleaching rate. It will be shown that morphological as well as
quantitative changes in actin organization of both NK and K562 cells occur during the cytotoxic process.

MATERIALS AND METHODS

Cells

NK cells, clone NK76 (generous gift from Dr. R.L.H. Bolhuis, Rotterdam), phenotype CD2 \(^{-}\)3 \(^{+}\)16 \(^{-}\)56 \(^{+}\), were cultured in 96-well plates in RPMI-1640 medium, supplemented with 25 mM Hepes, 2 mM L-glutamine, 10% pooled human serum, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 1 \(\mu\)g/ml indomethacin, 1 \(\mu\)g/ml leucoglutaminin (PHA-L), and 25 U/ml rIL-2. Cells were subcultured every seventh day on a layer of 30 Gy-irradiated feeder cells (Epstein-Barr-virus-transformed B cell lines APD and BSM, and PBL). K562 cells were maintained in exponential growth phase in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin (further referred to as a complete medium).

Conjugate Formation and Staining

Equal volumes of NK and K562 cells (2 \(\times\) 10\(^6\) cells/ml complete medium, total volume 100 \(\mu\)l) were mixed and centrifuged for 5 min at 200g. The cell suspension was gently resuspended and incubated at 37\(^\circ\)C for 0, 15, 30, 45, and 60 min. Control samples (consisting of NK and K562 cells, respectively, incubated alone) were treated in the same way. After incubation, cells were plated on poly-L-lysine-coated cover glasses (0.01\% PLL/PBS, overnight) and allowed to attach for 15 min at room temperature. The control samples and the sample consisting of both NK and K562 cells were plated on the same cover glass, divided by a thin layer of nail polish into three parts.

Cover glasses were rinsed twice with PBS and the cells were fixed with paraformaldehyde (3.7\% in PBS) for 15 min at room temperature. After fixation, cover glasses were rinsed twice with PBS and the cells were permeabilized with 0.1\% Triton X-100/PBS for 5 min at room temperature. After rinsing with PBS (twice), cells were labelled with a fluorescent derivative of phallolidin (rhodamine phallolidin or BODIPY 581/591 phallolidin; Molecular Probes, OR; 1 U per cover glass) for 40 min at room temperature. After rinsing with PBS (twice) cover glasses were mounted on the object glass in PBS + 50\% glycerol + 100 mg/ml DABCO (1,4-diazobicyclo[2.2.2]octane; anti-bleaching reagent) and sealed with nail polish.

Quantitative Fluorescence Microscopy

The inverted fluorescence microscope (IMT-2, Olympus Optical, Japan) was equipped with an intensified CCD camera (C2400-57), preprocessor (DVS 3000), and PC-Argus software, delivered from Hamamatsu Photonics, Germany. A filter block, consisting of a 530 nm long pass filter + a 545 nm band pass filter (exciitation), a 580 nm dichroic mirror, and a 590 nm long pass filter (emission) were used. The intensity of the excitation light was diminished using a neutral gray filter. The fluorescence light was collected using a 40 x oil-immersion objective, N.A. 1.0 (PlanApo 40 UV, Olympus Optical, Japan). Fluorescence images of the cells stained with rhodamine phallolidin were acquired by averaging 128 frames. Conjugates in which the cells were overlapping were not included in the analysis. Background images, acquired from a part of the cover glass without cells, were subtracted from the fluorescence images of the cells. The average fluorescence intensity was determined for each cell by defining individual cell borders. The values obtained were normalized over the mean intensity of the rhodamine phallolidin fluorescence of the control cells (NK and K562 cells, respectively, incubated alone).

Confocal Laser Scanning Microscopy

The confocal laser scanning microscope (CLSM; Leica Lasertecnik, Germany) was equipped with an inverted fluorescence microscope (Leica Fluovert FU) and an air-cooled argon-krypton laser. For the excitation of rhodamine phallolidin and BODIPY 581/591 phallolidin, the 565 nm laser line was used. A 580 nm long pass filter was used in the fluorescence detection path. The fluorescence was collected using a 63 x oil-immersion objective, N.A. 1.4 (PL Apo, Leica, Germany). Series of 2 x optically zoomed confocal sections, 1–2 \(\mu\)m apart, were scanned, each image averaged by 8 or 16 line scans. The images presented were obtained using the software function Simulated Fluorescence Process (SFP) (18).

Flow Cytometric Cytotoxicity Assay

The cytotoxic activity of NK cells was tested using the cytotoxicity assay developed in our laboratory (14) with slight modifications. Briefly, equal volumes of F-18 (octadecylamine-fluorescein isothiocyanate; cell membrane marker) labelled K562 cells and NK cells (2 \(\times\) 10\(^6\)/ml complete medium, total volume 50 \(\mu\)l) were mixed, centrifuged for 5 min at 200 g, and incubated for 0, 15, 30, 45, and 60 min at 37\(^\circ\)C. The control samples consisted of K562 cells incubated without NK cells. The samples were diluted with PBS, stained with propidium iodide (dead cell indicator), and analyzed by flow cytometer. The samples were measured in quadruplicate, and at least 4000 F-18+ cells were analyzed per measurement. The percentage of cytotoxicity was calculated for each incubation time as follows:

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\frac{(\text{cont} - \text{test}) \times 100}{\text{cont}} = \% \text{ cytotoxicity},
\]

where cont = number of F-18+, PI- cells (live K562 cells) divided by the total number of F-18+ cells (total number of K562 cells) in the control sample and test = number of F-18+, PI- cells (live K562 cells) divided by the total number of F-18+ cells (total number of K562 cells) in the test sample.
The relative F-actin content of interacting NK and K562 cells was determined after different incubation times (0, 15, 30, 45, and 60 min at 37°C) using rhodamine phalloidin (F-actin specific fluorescent probe) and quantitative fluorescence microscopy. Fluorescence images of the cells were acquired as described in Materials and Methods and analyzed for F-actin content.

Figure 1 shows the relative content of F-actin as a function of incubation time in conjugated NK and conjugated K562 cells, respectively. The mean intensity of rhodamine phalloidin fluorescence of the conjugated cells (NK and K562 cells, respectively) was normalized with respect to the mean intensity of rhodamine phalloidin fluorescence of the control cells (NK and K562 cells, respectively). As can be seen in the figure, there was a significant, incubation time dependent, increase of F-actin content in conjugated NK cells as well as in conjugated K562 cells. However, this increase showed different kinetics for NK and K562 cells. Already after the shortest incubation time (0 min incubation), conjugated NK cells exhibited two times higher F-actin content than control NK cells. This was followed by a slight increase upon incubation longer than 15 min. The F-actin content in conjugated K562 cells showed a maximum increase upon 15 min incubation, followed by a decrease upon incubation longer than 45 min. As can be learned from the figure, relative F-actin content in both conjugated NK and conjugated K562 cells increased during the cytotoxic process.

Morphology of F-Actin as Studied Using CLSM

Staining with fluorescent derivatives of phalloidin revealed two major pools of F-actin in control K562 cells (cells that were not incubated with NK cells). As can be seen in Figure 2A, bright fluorescence was observed in the cortical region, including microvilli, and in the cytoplasm. The cytoplasmic pool of F-actin was organized in aggregates, usually concentrated at one pole of the cell. In the control NK cells (Fig. 2B), F-actin was localized in the cortical region, with bright staining of pseudopodial extensions, while the rest of the cytoplasm showed diffuse staining.

During the cytotoxic process, concentration of F-actin in the NK cell towards the contact zone with the target cell was observed (Fig. 3A). More profound changes were observed in the organization of F-actin in conjugated K562 cells. Instead of the bright staining of microvilli and cytoplasmic aggregates, as observed in control K562 cells, filamentous F-actin structures could be observed in conjugated K562 cells (Fig. 3B). The filaments were sometimes oriented from the contact place with the NK cell towards the opposite pole of the K562 cell (Fig. 3C), forming a thread across the nucleus, with a somewhat more diffuse staining of the cytoplasm. In the phase of the cytotoxic process where blebs appeared on K562 cells, well-defined and loosely arranged F-actin filaments could be observed in the blebs, while in the cytoplasm more tightly packed F-actin filaments were localized (Fig. 3D). During the cytotoxic process nonconjugated K562 cells with filamentous F-actin structures appeared (Fig. 3E). These changes were not observed in control K562 cells (K562 cells incubated alone).

Figure 4 gives a schematic overview of the cell populations mentioned in this study. Figure 5 shows the behavior as a function of time of a fraction of the conjugated and the nonconjugated K562 cells, respectively, that showed filamentous F-actin structures. As can be seen from the figure, we found a time dependent increase for both fractions. However, different time dependences were observed. While the fraction of conjugated K562 cells with filamentous structures reached its maximum after 15 min incubation, followed by a slight decrease upon incubation of 45 min and longer, the fraction of nonconjugated K562 cells with filamentous structures showed a continuous increase with incubation time. The conjugated K562 cells with filamentous structures were further divided into cells where the filaments originated/ended at the contact place with the NK cell and cells where the filaments were organized randomly. Figure 6 shows the fraction of conjugated filamentous K562 cells without blebs that exhibited F-actin filaments oriented towards NK cells as a function of incubation time. As can be seen from the
figure, there was an incubation time dependent decrease in the fraction of conjugated filamentous K562 cells with orientated filaments.

Cytotoxic Activity

In order to correlate the observed changes in F-actin content and in morphology with NK cell activity, we have determined the cytotoxic activity at different incubation times. This was done using a flow cytometric cytotoxicity assay (14). As can be seen from Figure 7, the cytotoxicity (i.e., relative number of K562 cells killed) gradually increased with incubation time, indicating the effect of NK cell activity.

DISCUSSION

The cytotoxic activity of NK cells and CTL has been the subject of numerous studies. Different physiological parameters were investigated in order to define an exact mechanism by which these effectors of the immune system destroy their target cells. Different mechanisms have been proposed (4, for a review see 20), with the perforin model as the most accepted one (5,7,21). All of these models have the same feature: killer cell action causes an irreversible change in the target cell that leads to its death.

That the cytoskeleton may play an important role in the cytotoxicity was addressed in several studies (9,11,12). Intact microtubules have been shown to be needed for the final killing stage (12), while microfilaments seem to be more critical for conjugate formation and postlytic recycling of NK cells (9,11). Localization of F-actin in the killer cell towards the contact zone with the target cell was observed (2,3,15), leading to speculation about the role of killer cell microfilaments in the cytotoxic process. What is happening to microfilaments of cells that are attacked by NK cells has not been studied very extensively (2). Therefore we have concentrated on the organization of F-actin in NK cell sensitive target cells (K562) during the cytotoxic process.

Using quantitative fluorescence microscopy we detected an increase in the F-actin content in both conjugated NK and conjugated K562 cells (Fig. 1). This increase shows, however, different incubation time dependences for NK and K562 cells. While the conjugated NK cells underwent the largest increase in the F-actin content already upon 0 min incubation, the F-actin content in the conjugated K562 cells reached a maximum value upon 15 min incubation. The increase in the F-actin content in conjugated K562 cells occurs simultaneously with a morphological change of F-actin, as observed using confocal laser scanning microscopy. The poorly filamentous F-actin structures of K562 cells, consisting of the cytoplasmic aggregates of F-actin and F-actin rich microvilli (Fig. 2A), gives way to cytoplasmic filamentous structures (Fig. 3B—D).

Activation of different cell types has been correlated with the increase of relative F-actin content (1,6, 10,16). Therefore, we assume that the increase of F-actin content we observe in conjugated NK cells re-
Fig. 3. CLSM images of rhodamine phalloidin or BODIPY 581/591 labelled F-actin in conjugates. A: concentration of F-actin in the NK cell towards the K562 cell (top right, NK; bottom left, K562; SFP of four sections, 1 μm apart). B: F-actin filamentous structures in a conjugated K562 cell (top, NK; bottom, K562; SFP of two sections, 1 μm apart). C: F-actin filamentous structures in a conjugated K562 cell, filaments oriented towards NK cell (arrow; right, NK; left, K562; SFP of two sections, 2 μm apart). D: F-actin filaments in blebs (arrow) of conjugated K562 cell (bottom right, NK; up left, K562; one section). E: F-actin filamentous structures in non-conjugated K562 cells (top; “normal” K562; bottom, filamentous K562; SFP of three sections, 1 μm apart).
ACTIN IN CYTOTOXIC PROCESS

Fig. 4. Schematic overview of the cell populations mentioned.

Fig. 5. F-actin filaments. The fraction of K562 cells that exhibited morphological changes in F-actin (i.e., appearance of filamentous structures) was determined for different incubation time. At least 200 cells were analyzed for each incubation time. The error bars represent standard deviations of the measurements. Black bars, nonconjugated K562 cells; Shaded bars, conjugated K562 cells.

Fig. 6. Oriented F-actin filaments. The fraction of filamentous conjugated K562 cells without blebs that exhibited F-actin filaments oriented towards NK cells was determined for different incubation times. At least 100 cells were analyzed for each incubation time. The error bars represent standard deviations of the measurements.

reflects activation of the cells for the killing process. That the NK cells were active is proved by the presence of the cytotoxic activity, demonstrated using flow cytometric cytotoxicity assay (14) (Fig. 7). The increase of F-actin in conjugated K562 cells that occurs simultaneously with morphological changes of F-actin is a result of the attack of activated NK cells. The changes of F-actin organization in a conjugated K562 cell could be the result of damage to the cell by an NK cell. The other possibility could be that these changes are part of a mechanism by which the target cell tries to resist the attack of an NK cell (strengthening of the cell membrane?). It is also not excluded that these changes are induced by the NK cell itself as mechanism to aid the killing process. The orientation of F-actin filaments in K562 cells towards the contact zone with NK cells (Fig. 9C) could be attributed to the last two possibilities. The time dependent decrease in the number of conjugated filamentous K562 cells that exhibited F-actin filaments orientated towards NK cells (Fig. 6) could mean
that the orientation of F-actin towards the NK cell is an early stage of reorganization of actin in K562 cells attacked by NK cells. However, an exact time scale for the observed changes in the living cells as well as their role and importance in the cytotoxic process are factors that remain to be solved.

The decrease in a fraction of conjugated K562 cells with filamentous structures (Fig. 5) and the decrease in the relative F-actin content in conjugated K562 cells (Fig. 1) occur simultaneously with the increase in the fraction of nonconjugated K562 cells with filamentous F-actin (Fig. 5). These changes can be explained by detachment of NK cells from K562 cells after the cytotoxic interaction. Similarly, we observed in a study of membrane potential changes during the cytotoxic process (13), an incubation time dependent appearance of nonconjugated K562 cells that exhibit characteristics of conjugated K562 cells. Observation that F-actin concentrates in an NK cell towards the contact zone with the K562 cell is in agreement with previous reports (2,3,5). However, in only one of these reports was marginal reorganization of actin in the target cell reported (2).

Our study demonstrates the occurrence of profound changes in the content and organization of F-actin in tumor cells attacked by NK cells and raises questions about the role of target cell microfilaments in the cytotoxic process.

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**LITERATURE CITED**