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Serine protease detection in mixed lymphocyte cultures: a histochemical method for possible prediction of graft-versus-host disease

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Abstract

Background and Objective. **Graft-versus-host disease (GVHD) presents an important complication of allogeneic bone marrow transplantation. A method to predict GVHD might be the analysis of cytotoxic T lymphocyte precursors, but the technique requires the use of radioactive elements not suitable in all laboratories.**

Design and Methods. **Serine esterase (SE) activity was studied by a cytochemical method in donor-recipient mixed lymphocytes cultures (MLC). Twelve patients, affected by acute or chronic leukemia, and 20 donors were studied. MLC incubated with and without growth factors (IGF-1 or IL-2), were analyzed. The relationship between positivity of MLC and GVHD in transplanted patients was evaluated.**

Results. **The data obtained showed that the percentage of SE positive cells was higher in MLC compared to negative control MLC. The highest percentage of positive cells was found in the MLC obtained from unrelated subjects.**

Interpretation and Conclusions. **These results show that serine protease expression in MLC may be a predictive marker of GVHD and could be used as an additional early test associated with cytotoxicity.**
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Key words: GVHD, BLT-dependent serine-protease, mixed lymphocyte cultures

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells play very important roles in the immune system.[1,2] Serine protease activity appears to be involved in the cytotoxic mechanism in graft-versus-host disease (GVHD) following bone marrow transplantation (BMT).[3,4]

GVHD remains one of the most serious complications in allogeneic BMT.[5] Histocompatibility testing and MLC used to evaluate donor-recipient identity are not presently sufficient to predict the GVHD phenomenon. At the moment, the study of T lymphocyte precursor activation is the only reliable method to predict GVHD. This method employs radioactive substances such as ^{51}chromium (^{51}Cr) and is not commonly used in all laboratories.

Perforin and granzymes have been suggested as possible markers to identify functional cytotoxic lymphocytes due to a correlation between their expression and cytolytic capacity.[6] Cytoplasmic granules containing these enzymes appear in T lymphocytes after particular stimulation.[7] Recently, the presence of granzymes was shown in T lymphocytes isolated from cutaneous tissues of patients with GVHD and in peripheral blood of subjects with kidney allotransplants.[8-11]

In the present study, a cytochemical method[12,13] previously validated using peripheral blood lymphocytes, was applied using MLC to determine serine esterase (SE) activity. The aim of this study was to investigate whether these activities could predict the development of GVHD.

Materials and methods

Patients

Twelve leukemic patients (M/F: 8/4; mean age 35 yrs; range 24-48 yrs) were studied. Five patients were affected by chronic myeloid leukemia (CML); 4 patients were classified as having acute myeloid leukemia (M3) and 3 patients as acute lymphoid leukemia (L2) according to the FAB classification.

Mixed lymphocyte culture

Peripheral blood mononucleated cells (PBMC) were isolated by Ficoll-Hypaque gradient (1077 density) from heparinized peripheral blood. The cells were resuspended at a concentration of 1×10^6 cells/mL in RPMI 1640 medium supplemented with 20% human AB serum. Twenty mixed lymphocytes cultures (MLC) using donor plus recipient cells were set up according to the Dacie-Lewis method.[14] The results from positive and negative control cultures were compared along with donor/recipient matching (relative response, RR).

Positive controls included MLC using PBMC from

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6 non-correlated donors and donor plus pool or 3 different subjects. The cultures were incubated for 6 days at 37°C in a 4% CO_2 atmosphere. In addition freshly isolated PBMC from healthy donors, human SE-deplete epithelioid line Chang (ATCC CL13) lacking SE activity and PBMC treated with interleukin-2 (IL-2, 50 pg/mL) or insulin growth factor-1 (IGF1, 1 ng/mL) were used.

Cytochemical detection of serine esterase activity

Smears obtained from each MLC were processed for the detection of serine esterase (SE) activity. Before fixation in 1% (w/v) paraformaldehyde solution, the samples were dried for 1 min and then washed in phosphate-buffered saline, pH 7.2 for 1 min.

The serine protease assay was performed on smears according to Wagner's method[12,13] using N-a-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) (Sigma) as the substrate. Following incubation, the smears were washed in tap water and then counterstained with Carazzi hematoxylin for 10 min.

Serine esterase activity was evaluated by calculating the percentage of cells showing intracytoplasmic dark granules; 500 cells were analyzed from each culture. The percentage of positive cells in (R*+D) (R* = *irradiated recipient; D=donor), (R+R*) and (D+D*) control cultures were determined.

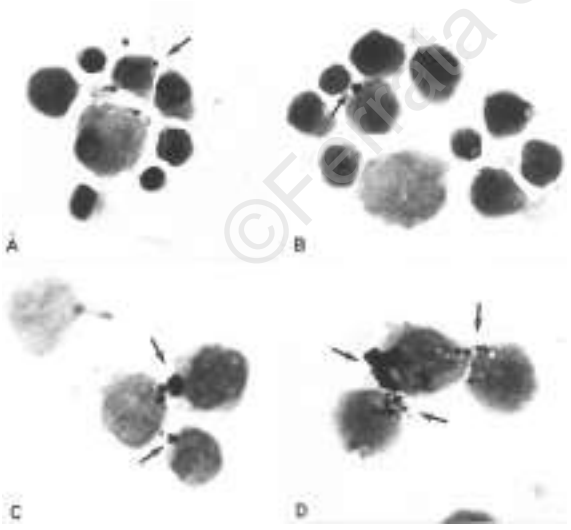

Figure 1. Serine esterase (SE) cytochemical reaction of mixed lymphocyte cultures. Dark granules as the reaction product of the BLT/Fast Blue BB substrate/chromogen reaction mixture, are observed: A) in a granular spread pattern; B) presence of one large dark and many small granules localized in the cytoplasm of the cells; C) strong SE reaction with positioning of large granules toward a target cell; D) increase of staining intensity near the effector/target interspace (×1200).

Statistical analysis

Results were reported as mean values ± standard error. Statistical comparison was performed by means of the Fisher's exact test.

Results

Serine esterase positivity was studied in 20 MLC using PBMC from 12 leukemic patients and 20 HLA compatible donors. The cells from positive (D+P*) and negative (D+D*) control cultures were compared using light microscopy; dark granular products of the enzymatic reaction were localized adjacent to the cytoplasmic membrane, in the intercellular and pericellular area of the lymphocytes. Moreover, granules were seen in the culture medium when extrused by cytotoxic cells. In the cells lacking granzymes and in the cells of the MLC (D+D*) negative controls, dark granules were not observed.

Figure 1 shows patterns of SE positive cells observed in MLC. The percentage of positive cells in the different cultures was calculated using at least 500 cells from each culture. In the (D+P*) MLC, the percentage of SE positive cells was significantly higher than the negative controls (D+D*) (1.54±0.07 vs 0.83±0.13; $p < 0.05$).

The data obtained for each donor-recipient MLC were compared with the mean values of the negative and the positive controls (Figure 2). In 14/20 (D+R) MLC, the percentage of SE positive cells was higher than the mean value from negative control cultures. In 6/20 (D+R*) MLC cultures, the number of positive cells was higher than the mean value from positive control cultures.

The number of serine protease positive cells was also higher in the MLC of unrelated HLA-identical donors than in related one ($p < 0.05$) (1.7±0.2 vs 1.3±0.2; $p < 0.05$). Thirteeen out of 18 (72%) of the donors were histocompatible for transplantation, with RR values being < 10. Table 1 shows SE positivity and the outcome of transplantation of the 12 leukemic patients studied. Ten patients received bone marrow from a compatible donor; 2 patients were transplanted using donors with RR values >10. GVHD occurred in 5/12 (41%) patients: 2 with grade I, 2 with grade II and 1 with grade III (cutaneous and hepatic graft) disease. The MLCs of 3 of the patients showed a significant percentage of SE positive cells ($P<0.05$). All 3 patients developed GVHD (1 with grade I, 1 with grade II, 1 with grade III).

To optimize the experiment, a study using various negative and positive controls was extended. These included freshly isolated lymphocytes from healthy donors, a cultured cell line lacking SE activity (Chang) and MLC stimulated by either IL-2 or IGF-1.

The results are summarized in Figure 3. Both freshly isolated lymphocytes and the Chang cell line showed negligible positivity. However, a significant increase of SE positive cells was observed in lymphocytes, from a single donor, stimulated by IL-2 for 6

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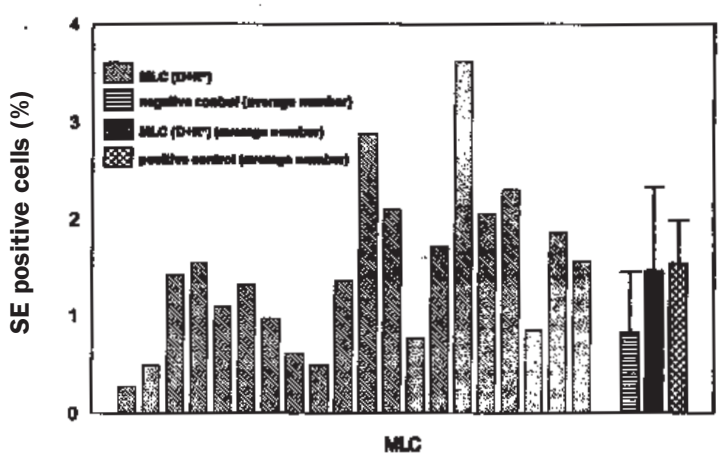

Figure 2. The percentage of SE positive cells in all CML are compared with the average value of negative and positive controls. At least 500 cells from each sample were studied.

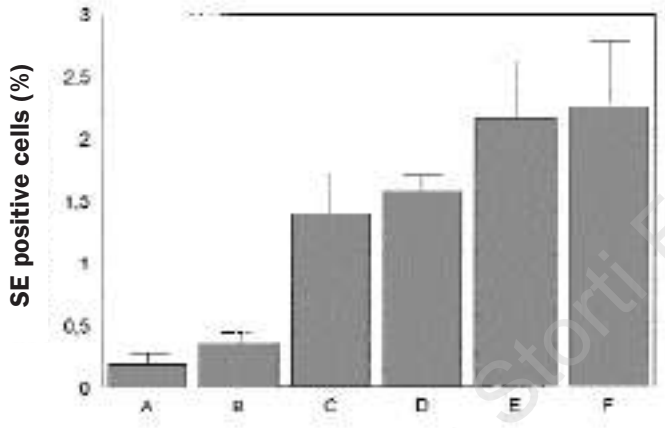

Figure 3. Serine esterase positivity: percentage of positive cells in various types of cells. Peripheral blood lymphocytes from 4 healthly donors (A); human cell line Chang (B) (average from 5 different samples); lymphocytes from 4 healthy donors stimulated with IL-2 (C); MLC from 6 unrelated donors (D); MLC from 6 unrelated donors stimulated with IGF-1 (E); MLC from 6 unrelated donor stimulated by IL-2 (F).

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days (C) ($p < 0.05$) and in 6 MLCs from unrelated donors (D). The addition of the growth factors IGF-1 (E) and IL-2 (F) increased the percentage of positive cells. IL-2 addition resulted in a higher percentage of SE -positive cells than IGF-1.

Discussion

Granzymes are a family of highly homologous serine proteases (of the serine esterase category) which have a function in cell-mediated lysis.[15]

Various kinds of granzymes, recognized by specific monoclonal antibodies, have been found.[7,9,19,20] High levels of SE activity have been demonstrated in cytotoxic T lymphocytes in the lymphoid tumoral cell line.[6] It has been shown that after 5 days there is a significant association between cytotoxic activity and enzyme activity in murine chronic myeloid leukemia.[6] In cytotoxic T lymphocytes, proteases are located within the cytoplasmic granules[16] and are associated with pore-forming proteins known as perforines. Following perforin polymerization, membrane lesions form in the lipid layer of the target cells.[17,18]

In this present study a cytochemical method[12,13] was applied in order to detect SE activity in cells of MLC with the aim of correlating enzyme activity with GVHD.

The activation of granzymes in MLC can derive from various GVHD grade differences between donor and recipients with unknown histocompatibility determinants. GVHD patients showed both a cutaneous lymphocytic infiltrate rich in granzyme positive cells and T cell lymphocyte activity and this encouraged us to ask whether these enzyme activities may be predictive markers of GVHD.

The data obtained showed that the percentage of SE positive cells was higher in MLC (D+R*) compared to negative control MLC (D+D*). The highest percentage of positive cells was found in the MLC obtained from unrelated subjects. Following the addition of growth factors to MLC, an increase of SE activity was observed as a possible consequence of clonal expansion of cell subpopulations with specific receptors.[12,21]

The effects of 2 different growth factors were studied; somatomedin (IGF-1), whose receptors are present on nucleated cells, and IL-2, whose receptors

Table 1. Serine esterase positivity and post–transplantation outcome in 12 leukemic patients.

Patients	Sex/Age	Pathology	SE positivity	BMT	GVHD
1	M/29	AML (M3)	–	R	–
2	M/41	AML (M3)	–	R	+ (II)
3	M/37	ALL (L2)	–	R	–
4	M/31	AML (M3)	–	R	–
5	F/32	ALL (L2)	+	R	+ (III)
6	M/41	AML (M3)	–	R	+ (I)
7	F/25	LNH	+	R	+ (I)
8	F/48	CML	–	R	–
9	M/34	CML	+	R	+ (II)
10*	F/24	CML	–	U	–
11*	M/42	CML	–	U	–
12	M/33	CML	–	R	–

**RR > 10; +(SE positivity) p > 0.05; R: related; U: unrelated.*

are expressed on T cell activated lymphocytes and in NK/LAK cells. The results showed that IL-2 is more efficient than IGF-1 in inducing the expression of granzymes. The relationship between SE positivity of MLC and GVHD in patients transplanted with a bone marrow selected according to the relative responses was evaluated. Three out five patients that progressed to GVHD showed a significant increase of SE in MLC.

According to these data, SE positivity in MLC might be predictive of GVHD.

Further BMT samples are being analyzed in order to extend the investigation.

Contributions and Acknowledgments

MAM performed the cytological analysis; RN revised critically the paper and gave the final approval for publication; EC was the principal responsible for the conception of the study. MB, EPA, PB were the clinicians involved in the follow-up of the patients.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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