Transient Versus Permanent Expression of Cancer-Related Glycopeptides on Normal Versus Leukemic Myeloid Cells Coinciding With Marrow Egress

By Wim van Beek, Abraham Tulp, Jan Bolscher, Geke Blanken, Klaas Roozendaal, and Mieke Egbers

Release of mature cells from the bone marrow (BM) into the peripheral blood (PB) compartment is supposed to be triggered by changes in cell surface constituents, most probably in glycoproteins. The supposed importance of glycoproteins in marrow exit prompted us to investigate glycopeptides, i.e., the carbohydrate part of the cell-surface-located glycoproteins of isolated human bone marrow cells of the myeloid series at different stages of maturation. Fractionation of cells was performed by a four-step procedure, comprised of density gradient centrifugation and velocity sedimentation at unit gravity in specially designed separation chambers. With this method, promyelocytes/myeloblasts, granulocytes from bone marrow, and granulocytes from peripheral blood were isolated in high quantity with purities up to 90%, 90%, and 100%, respectively. Surface glycopeptides of the various myeloid cells were investigated by gel filtration analysis after metabolic labeling with radioactive fucose or after external labeling with periodate-borotritide under mild conditions. Within the normal myeloid maturation sequence, mature granulocytes within the bone marrow were found to transiently express altered surface glycopeptides, which disappeared after release into the peripheral blood. These oligosaccharide structures appeared similar to those encountered on leukemic blast cells, known as "cancer-related glycopeptides." In contrast to normal granulocytes from BM, leukemic blast cells retained these aberrant carbohydrate structures on their surface after marrow release. A possible role for cancer-related glycopeptides in the process of marrow cell exit might be hypothesized.

A DULT MAMMALIAN hematopoiesis is located in the extravascular spaces between the marrow sinuses. Mature cells enter the circulation by passing through the walls of these sinuses, which are composed of a luminal layer of endothelial cells and an abluminal coat of adventitial reticular cells. 1-3 Reticular cells in the bone marrow are anatomically related to immature hematopoietic cells, suggesting a functional as well as a structural relationship. Intimate cell contacts between reticular cells and immature hematopoietic cells are considered to be necessary for the differentiation, proliferation, and possibly maturation1-3 and are supposed to act as an anchor, reducing the possibility of their discharge from the marrow. 1 These cell contacts could be observed by using tannic-acid-glutaraldehyde fixation, indicating that glycoproteins rather than glycosaminoglycans are involved. 1 Marrow cells enter the lumen of the sinus through pores at the parajunctional zone of the endothelial cell, which are formed at the time of migration and seal when migration is complete. 8 Selectivity in release of mature cells would supposedly be guaranteed due to the increased ability of mature cells to "bore" holes and translocate more efficiently than immature cells. 9 This ability seems to reside at the cell periphery and is based on cellular interactions that mediate the delivery of blood cells into the circulatory system. 10,11 The apparent importance of glycoproteins in cellular interactions possibly controlling marrow cell release 7 prompted us to investigate the carbohydrate part of cell-surface-located glycoproteins, i.e., fucosyl glycopeptides of isolated human bone marrow cells of the myeloid series at different stages of maturation. Findings were compared with observations in leukemic disease.

MATERIALS AND METHODS

Control granulocytes and monocytes were isolated from the peripheral blood of patients ready for cardiac surgery and from normal buffy coat blood, respectively. Peripheral blood was freed from the bulk of erythrocytes by dextran-mediated sedimentation at unit gravity12,13 for 10 min at room temperature in a chamber developed by Tulp et al.,14 using Maxidens (Nyegaard, Oslo, Norway) as a cushion liquid. The supernatant cells were subsequently separated by density gradient centrifugation at 90 g in a newly designed separation chamber, as described before.14,15 The velocity sedimentation chamber is commercially available (Phywe, Göttingen, Germany). Granulocytes and monocytes from specific fractions were cultured overnight in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS), antibiotics (penicillin 100 IU/ml, streptomycin 50 µg/ml), and L-[1-3H]-fucose (5-10 µCi/ml; 2.8 Ci/m mole, New England Nuclear, Boston, MA) or L-[1-14C]-fucose (1-2 µCi/ml; 60 Ci/mole, Radiochemical Centre, Amersham, Bucks, England) at cell densities of 5 × 10⁶ and 10⁷ cells/ml, respectively, in a humidified incubator at 37°C in 5% CO₂. These incubation conditions were used for all different cells investigated and at cell densities of 5 × 10⁶ cells/ml unless stated otherwise.

Immature cells of the granulocyte series were isolated from bone marrow obtained by aspiration from the sternum of patients ready for cardiac surgery, as described earlier.15 Debridinated bone marrow (BM) cells were washed with phosphate-buffered saline (PBS) and resuspended in 60% (v/v) Percoll at room temperature. A linear gradient, ranging from 60% to 37.5% Percoll, was introduced into

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Submitted January 7, 1983; accepted July 30, 1983.

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0006-4971/84/6301-0021$01.00/0

the separation chamber, followed by an overlay of 22 ml 25% Percoll. Centrifugation was for 60 min at 600 rpm (90 g) in a Kontron TGA6 centrifuge at room temperature and at atmospheric pressure. Fractions of 4 ml each were collected, and the fractions containing the majority of immature cells of the myeloid series were combined (resulting in fraction II–V, Fig. 1) and used for the investigation on fucosyl glycopeptides. The promyelocyte-enriched fraction (fraction I, Fig. 1) was further separated after washing with PBS at 4°C. The majority of the erythrocyte precursor cells were lysed by a hypotonic shock, essentially as described by Fallon et al.

The remaining cells were resuspended in PBS and layered onto a linear 1%–7% (w/v) bovine serum albumin (BSA) gradient in a unit gravity sedimentation chamber as described. Total time from layering to complete fractionation was 2 hr. The temperature was 4°C. Fractions of 10 ml each were collected, and the number and size of cells was measured in a Coulter counter (model ZF) equipped with a channelizer (Tracor TN 1705). The promyelocyte-rich fraction was washed with PBS at room temperature and resuspended in RPMI 1640 medium, containing 20% FCS, and incubated for 1 hr at 37°C in a glass Petri dish to eliminate contaminating monocytes.

Nonadhering cells, representing predominantly promyelocytes, were incubated in culture medium supplemented with radioactive fucose at a cell density of 0.5–1 x 10^6 cells/ml. In some experiments, external labeling of sialic-acid-containing residues on the cell surface of BM- and peripheral blood (PB)-derived granulocytes was performed by means of the periodate-borotritide method, as described by Flowers and Glick.

Leukemic blast cells from PB and BM of patients V.A. and R.O., suffering from a relapse of acute myelomonocytic leukemia (AM-MoL) and acute undifferentiated leukemia (AUL), respectively, were isolated by density gradient centrifugation at 90 g just before (renewed) administration of cytotoxic drugs. Isolation of blast cells and incubation with radioactive fucose was essentially as described for normal cells. Cell morphology was examined with May-Grunwald-Giemsa stain (Merck, Darmstadt, Germany). Slides of suspension cells were prepared by using a Shandon-Elliot cytospin centrifuge. For each determination, 500 cells were counted. Flow cytometric determination of DNA content per cell was performed on ethidium-bromide-stained cells, as described by Smets et al.

Radioactive fucose or externally labeled glycopeptides were isolated by trypsin treatment of cells. The supernatant was further digested by pronase (Calbiochem, La Jolla, CA, B-grade), desalted by gel-filtration–centrifugation on Biogel P2, and subsequently analyzed on 100x1 cm Biogel P10, 200–400 mesh—Sephadex G50 superfine (2:1), or on a 100x1 cm Biogel P30 fine column as described earlier.

Mild acid treatment of the glycopeptides was performed by incubating differentially labeled test and control glycopeptides in a waterbath at 80°C for a period of 90 min at pH 2.0, as described earlier.

**RESULTS**

Normal BM cells were separated according to their buoyant density. The density profile was marked by several peaks and shoulders of peaks (Fig. 1). The obtained pooled fractions, denoted I–V (Fig. 1), dif-

![Fig. 1. Equilibrium density centrifugation of human bone marrow (BM) cells. BM cells were washed and resuspended in 60% (v/v) Percoll. A linear gradient, ranging from 60% to 37.5% Percoll, was introduced into the separation chamber on top of a cushion of 80% Percoll followed by an overlay of 22 ml 26% Percoll. Centrifugation was for 60 min at 600 rpm (90 g) in a Kontron TGA6 centrifuge at room temperature and at atmospheric pressure using a slow acceleration–deceleration schedule. Fractions of 4 ml each were collected. The differential count of cells in the pooled fractions, denoted I–V, are mentioned in Table 1. The bulk of erythrocytes was collected immediately after fraction V.](image-url)

**Table 1. Differential Count of Separated Normal Bone Marrow (BM) and Peripheral Blood (PB) Cells**

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Myeloblasts and Promyelocytes</th>
<th>Myelocytes</th>
<th>Metamyelocytes</th>
<th>Granulocytes</th>
<th>Bands</th>
<th>Segments</th>
<th>Monocytes and Macrophages</th>
<th>Lymphocytes and Plasma Cells</th>
<th>Other Cells</th>
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<tbody>
<tr>
<td>BM 90gl</td>
<td>35.6</td>
<td>3.0</td>
<td>0.4</td>
<td>1.0</td>
<td>6.0</td>
<td>24.2</td>
<td>26.2</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>BM 1gI V</td>
<td>86.4</td>
<td>4.2</td>
<td>0</td>
<td>0.6</td>
<td>1.6</td>
<td>2.2</td>
<td>1.2</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>BM 90gl</td>
<td>6.4</td>
<td>9.6</td>
<td>53.0</td>
<td>19.4</td>
<td>6.0</td>
<td>0.4</td>
<td>3.4</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>BM 90gII</td>
<td>2.0</td>
<td>12.0</td>
<td>41.6</td>
<td>37.4</td>
<td>6.2</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>BM 90gIV</td>
<td>0.4</td>
<td>1.0</td>
<td>6.2</td>
<td>26.2</td>
<td>65.4</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>BM 90gV</td>
<td>0</td>
<td>0.4</td>
<td>2.6</td>
<td>19.8</td>
<td>76.2</td>
<td>0</td>
<td>0.8</td>
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<tr>
<td>PB 90gl</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0</td>
<td>3.0</td>
<td>90.0</td>
<td>7.0</td>
<td>0</td>
<td></td>
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<tr>
<td>PB 90gIli</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.2</td>
<td>98.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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</tbody>
</table>

Normal cells were isolated from aspirated bone marrow and peripheral blood of patients ready for cardiac surgery. Cells were separated by density gradient centrifugation, as described before. The different fractions varied in content of mature and immature myeloid cells. The promyelocyte-enriched fraction (BM 90gl) was further separated by means of a four-step isolation procedure comprised of velocity sedimentation at unit gravity in specially designed separation chambers, as described earlier.

Slides of suspension cells were prepared by using a Shandon-Elliot cytospin centrifuge. Cell morphology was examined with May-Grunwald-Giemsa stain on 500 cells each. The cell preparations listed in this table correspond to the ones used for glycopeptide analysis in Figs. 2 and 3. Numbers represent percentages.
ferred in the amount of mature and immature myeloid cells (Table 1). The low-density part of the gradient (Fig. 1, fraction I) contained immature myeloid precursor cells (Table 1, BM 90g1), which were subjected to further treatment and separated as described in Materials and Methods. Fraction II of Fig. 1 contained largely metamyelocytes, fraction III metamyelocytes and banded cells, fraction IV banded and segmented cells, while fraction V contained mostly incompletely segmented cells (Table 1). PB-derived granulocytes were fully matured, containing nuclei with several segmentations, in contrast with the less mature BM-derived granulocytes. This difference in morphology permitted the estimation of PB contamination of the BM aspirates, which appeared to be minor in the different experiments performed. For the various glycopeptide analyses, BM- and PB-derived granulocytes were obtained from the same donor. Promyelocytes of very high purity were isolated, as shown for a representative experiment in Table 1 (BM 1gIV). The number of promyelocyte isolations, as well as the other normal cell isolations, amounted to 7, and the promyelocyte/myeloblast purity of the different preparations varied from 78.4% to 90.6% (mean 85.7%). These promyelocyte fractions contained 16.0%–27.4% (mean 22.6%) S-phase cells, which further increased to 26.0%–44.0% (mean 32.6%) upon incubation in culture medium overnight. The latter percentage of S-phase cells was comparable with that of growing promyelocytic leukemia HL-60 cells.  

Cells were differentially labeled with 3H- or 14C-fucose in order to subject the glycopeptides of control cells and cells under investigation to exactly identical conditions for digestion and analysis. Fucose is a specific label for glycoproteins and is not catabolized in the general metabolic pool.  

Extensive pronase treatment of the trypsinate of cells resulted in glycopeptides that consist of an oligosaccharide portion with 3–5 amino acids attached. Analysis was performed by gel filtration, which is known to be a powerful technique for separating oligosaccharides or glycopeptides.  

Investigation of fucosylglycopeptides of the various normal myeloid cells, varying in the degree of maturation, revealed that mature granulocytes from BM (Table 1, BM 90g1V–V) exhibited distinct glycopeptides, as shown in Fig. 2A (fraction 42–49). The latter glycopeptides eluted considerably faster on a gel filtration column than those of PB-derived granulocytes and BM-derived promyelocytes.  

This deviating elution behavior is not caused by differential release of glycoproteins from the cell surface by trypsin. In agreement with earlier results, about 25% of the fucose-containing macromolecules was digested from the different cell surfaces, while stripping of glycoproteins by means of pronase—a mixture of proteolytic enzymes—instead of trypsin produced identical results. External labeling of sialic-acid-containing molecules on the surface of mature granulocytes from BM and PB by periodate-borotri-

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**Fig. 2.** Elution profile of surface fucosyl glycopeptides from mature BM-derived granulocytes (Table 1, BM 90gV) (●), mature PB-derived granulocytes (Table 1, PB 90gIII) (○), and normal immature promyelocytes (Table 1, BM 1gIV) (△), before (A) and after (B) mild acid treatment. Cells were differentially labeled with [3H]- or [14C]-l-fucose or vice versa. Cell trypsinites were pronase digested, further processed as described in Materials and Methods, and subsequently co-chromatographed on a Biogel P10-Sephadex G50 (2:1) column. The promyelocyte profile was drawn into this figure by comparison with a common PB-derived granulocyte profile. BD, Blue Dextran-2000.
GLYCOPEPTIDES IN MARROW EGRESS

tide\textsuperscript{19} resulted in glycopeptide size distribution patterns comparable to those obtained with metabolic labeling, revealing the same characteristic shift to higher apparent molecular weight glycopeptides of the BM-derived granulocytes as compared with those of PB-derived granulocytes (Fig. 3A). In agreement with the findings of Glick et al.\textsuperscript{19} on BHK cells, the larger glycopeptides were enriched using external sialic acid labeling as compared with metabolic fucose labeling (compare Fig. 3, A and B).

The aberrant glycopeptides were partially resistant to mild acid treatment, known to split off exclusively sialic acid\textsuperscript{22} (Fig. 2B, fraction 42–59), emphasizing their different glycopeptide structure as compared with those of normal promyelocytes and PB-derived granulocytes. This finding also indicates that differences in sialic acid content are not the sole cause of the observed change in glycopeptides. The presence of these fast eluting glycopeptides on the surface of BM-derived granulocytes, and to a much lesser extent on cells of myelocyte or metamyelocyte containing preparations (not shown), indicates that these oligosaccharide structures appear at the normal cell surface at a distinct point along the myeloid maturation sequence, presumably between the metamyelocyte and the banded cell stage of development.

Surface glycopeptides of highly purified normal promyelocytes, as compared to those of granulocytes of PB, eluted slightly slower on the gel and were enriched in the relatively smaller sialic-acid-devoid glycopeptides (Fig. 2A, fractions 45–52 and 61–85, respectively). The latter result deviates somewhat from our earlier observation,\textsuperscript{15} a difference that can be explained by the present use of highly purified promyelocytes. The various elution profiles were repeated twice and were affected neither by using radioactive \(^{3}H\) instead of \(^{14}C\) label or vice versa nor by altering the incubation time for radioactive metabolic labeling from 16 hr to 3 hr, nor by varying the cell density during labeling from \(10^6\) cells/ml to \(10^7\) cells/ml, nor by loading the column with different amounts of glycopeptides, in agreement with earlier results.\textsuperscript{22}

The elution profile of normal PB-derived granulocytes was more or less comparable with that of immature myeloid cells (Fig. 2). Thus, the fast eluting glycopeptides on the surface of BM-derived granulocytes apparently disappeared after BM egress (Fig. 2). In contrast, leukemic blasts did not "normalize" their surface glycopeptides upon BM egress, as shown in Fig. 4. Leukemic blast-cell-derived glycopeptides from BM of patient V.A. (purity 83.2%) almost coincided with those derived from PB (purity 92.8%) and both
contained fast eluting glycopeptides as compared with normal control cells (Fig. 4, fraction 40–50). A similar result was observed with leukemic blast cells of BM (purity 92.5%) and PB (purity 91%) of patient R.O., who was suffering from acute undifferentiated leukemia.

DISCUSSION

The present data show that alterations in fucosyl glycopeptides were present when normal myeloid cells mature to granulocytes ready for marrow egress. These alterations were caused by the generation of large-size glycopeptides on the cell surface of BM-derived mature granulocytes. In contrast, PB-derived granulocytes, and to a larger extent, immature promyelocytes from normal BM, possess normal and considerably smaller-sized glycopeptides. Studies with fibroblasts in tissue culture have shown growth-dependent differences in the glycopeptide distribution pattern. Growing fibroblasts appeared relatively enriched in larger structures as compared with those of plateau-phase cells. It is unlikely that glycopeptide differences observed between promyelocytes (32% S phase) on the one hand, and mature PB- or BM-derived granulocytes (<1% S phase) on the other hand, are caused by differences in growth capacity for the following two reasons. First, no growth-dependent changes were observed in hematopoietic cells, in deviation from the results with cells from other morphology. Second, promyelocytes appeared to expose relatively smaller-sized glycopeptides instead of the “growth-related” larger structures. The dramatic change in the glycopeptide size distribution pattern of BM-derived granulocytes, as compared with PB-derived granulocytes, was not restricted to fucosyl glycopeptides, but was also observed in sialoglycopeptides, as determined by external labeling using periodate-borotritide immediately after isolation of cells (Fig. 3A). We can therefore conclude that these altered oligosaccharides were neither the result of the in vitro condition nor the reflection of precursor pool differences that might exist among the various cell types studied. Mild acid treatment of these oligosaccharide structures of BM-derived granulocytes revealed that, in addition to sialic acid, supplemental neutral monosaccharides were responsible for the larger apparent molecular weight. This might explain why the difference in glycopeptide size distribution pattern between BM and PB granulocytes was less when labeled by periodate-borotritide than by fucose (compare Fig. 3, A and B). The present finding could very well correspond with the observation that the density of sialylated glycoproteins increases in the membrane of the differentiating erythroid cell of the rat, but contrasts to the finding of Lightman and Weed. The latter authors reported a decrease in cell surface sialic acid density upon differentiation along the myeloid series. However, these investigators measured differences in the total neuraminidase-susceptible sialic acid of the cell surface by alterations in cell electrophoretic mobility, while using relatively impure BM cell preparations. The large-size glycopeptides on the surface of BM-derived granulocytes (Fig. 2, fraction 42–49) appear to be very similar to those found on leukemic blast cells of patient V.A., suffering from AMMoL (Fig. 3, fraction 40–50), of patient R.O., suffering from AUL, and on promyelocytic leukemia HL-60 cells in particular. Both HL-60 cell glycopeptides and BM-derived granulocyte glycopeptides (Fig. 2B) exhibited similar partial resistance to mild acid treatment, also indicating a possible structural resemblance. Studies using high resolution 1H-NMR spectroscopy are presently being performed to establish this possible structural similarity.

The presence of glycopeptides with a larger apparent molecular weight on the surface of granulocytes of normal bone marrow is surprising. Until recently, oligosaccharides of this type were found exclusively on the surface of malignant or premalignant cells and were referred to as “A group glycopeptides,” “fast eluting glycopeptides,” or “cancer-related glycopeptides” and their relation to the malignant phenotype of cells was confirmed by several laboratories. This shift to larger apparent molecular weight was not dependent on fucose labeling and was also obtained with monosaccharides other than L-fucose or by external labeling using periodate-borotritide, as shown by Glick et al. for control and virally transformed BHK cells. A few exceptions were reported recently. Whereas glycopeptides of tumor-derived renal epithe-


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Transient versus permanent expression of cancer-related glycopeptides on normal versus leukemic myeloid cells coinciding with marrow egress

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