MORPHOLOGICALLY NORMAL, CD30-NEGATIVE B-LYMPHOCYTES WITH CHROMOSOME ABERRATIONS IN CLASSICAL HODGKIN’S DISEASE: THE PROGENITOR CELL OF THE MALIGNANT CLONE?

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SUMMARY

A recent study observed that numerical chromosome abnormalities in Hodgkin’s disease (HD) are detected not only in morphologically abnormal Hodgkin/Reed–Sternberg cells, but also in a fraction of morphologically normal cells. However, the phenotypic constitution of these genetically abnormal, morphologically normal cells and their relationship to the malignant Hodgkin/Reed–Sternberg cells could not be established in the earlier cases studied, because of the low frequency of these cells. The present study investigated two cases of classical Hodgkin’s disease containing a relatively large population of such apparently normal cells with aberrant chromosome copy numbers. The phenotype and their position within the developmental route of the malignant compartment were examined by a combined in situ hybridization and immunocytochemistry approach. Numerical abnormalities for chromosome 1 in one case and for chromosomes X, Y, and 1 in the other case were observed not only in CD30-positive Hodgkin/Reed–Sternberg cells, but also in CD30-negative, morphologically normal cells. It was shown that these genetically aberrant cells expressed the B-cell antigen CD19, thus confirming their B-cell nature. These studies indicate a relationship between the genome aberrations in these genetically abnormal, morphologically normal B-cells and the Hodgkin/Reed–Sternberg cells, suggesting that they are progenitor cells of the malignant cell fraction. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—Hodgkin’s disease; progenitor cell; B cells

INTRODUCTION

Studies on the cell biology of Hodgkin’s disease focus in general on the small population of Hodgkin/Reed–Sternberg cells, in an attempt to clarify the origin of these tumour cells. Structural and numerical chromosome aberrations are observed in these cells using karyotyping and in situ hybridization procedures, but no specific chromosome abnormality could be found to characterize Hodgkin’s disease.1–6 Furthermore, V H- and V L-gene rearrangement analysis of single cells has demonstrated that Hodgkin/Reed–Sternberg cells are monoclonal and have a B-cell phenotype.7–11 However, in cases of Hodgkin’s disease analysed by flow cytometry, the fraction of aneuploid cells was larger than the fraction of Hodgkin/Reed–Sternberg cells.12

We recently reported that chromosome abnormalities in Hodgkin’s disease are not restricted to the Hodgkin/Reed–Sternberg cells.13 Small populations of morphologically normal cells (defined as lymphocytes without characteristics of Hodgkin cells or Reed–Sternberg cells) in malignant lymph nodes were shown to exhibit numeric-
originally diagnosed in 1991. A cell line (L1236) could be established from atypical mononuclear lymphocytes of the peripheral blood of this patient. Paraffin sections (4 μm thick) of the bone marrow specimen obtained for monitoring tumour progression at the time of establishing the cell line were analysed by combined immunocytochemistry and in situ hybridization.

Case 2—In December 1997, a male patient (age 68 years) was diagnosed with nodular sclerosing Hodgkin's disease. A fresh lymph node biopsy was used for routine pathological and cytogenetic analysis. The pathological analysis included CD20 and CD30 staining of the sections. For the cytogenetic analysis, cell suspensions obtained from this biopsy and from peripheral blood were cultured in RPMI1640 containing 15 per cent fetal calf serum and 1 per cent glutamine. Cultures were incubated in a CO2-incubator for 48 or 72 h when stimulated with pokeweed or phytohaemagglutinin. Karyotyping was based on G-banding after trypsin–Giemsa staining.

CD19, CD20, and CD30 immunocytochemistry

Paraffin sections (4 μm thick) from case 1 were mounted on Super Starfrost Plus glass slides (Menzel Glaeser, Germany) and CD30 immunocytochemistry was performed according to the manufacturer’s protocol (DAKO A/S, Glostrup, Denmark). The anti-CD30 antibody (BerH-2) was detected with biotin-labelled horse anti-mouse IgG (Vector, Brunschwig Chemie, Amsterdam, The Netherlands) diluted 1:200, followed by avidin/biotinylated peroxidase (Vector), both diluted 1:50. The sections were then counterstained with haematoxylin and peroxidase activity was subsequently detected with 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma, St. Louis, MO, U.S.A.) and H2O2 as described previously by Speel et al. Haematoxylin staining prior to the TMB reaction is required to improve the stability of TMB precipitate.

Cell suspensions from a fresh lymph node biopsy of case 2 were cytospun for 5 min at 700 rpm onto Super Starfrost Plus glass slides (Menzel Glaeser). These preparations, as well as frozen sections (4 μm thick), were immunologically stained for CD19, CD20 or CD30 (DAKO A/S) and subsequently pretreated for in situ hybridization. The protocol for this combined procedure has been described by Weber-Matthiesen et al.

In situ hybridization

A biotinylated DNA probe for the (peri)centromeric region of chromosome 1 (pUC1.77, 1-77 kb) was prepared according to standard procedures. Furthermore, directly fluorochrome labelled DNA probes for the (peri)centromeric regions of chromosomes 1, X (pBamX5, 2.0 kb) and Y (DYZ1, 2.1 kb; recognizing the long arm of Y chromosome) were prepared according to the instructions of Boehringer Mannheim, Mannheim, Germany. These DNA probes were labelled with FITC-11-dUTP (Boehringer Mannheim) or rhodamine-4-dUTP (Amersham, Buckinghamshire, U.K.).

The 4 μm thick paraffin sections were pretreated with 85 per cent formic acid/0.3 per cent H2O2 (Merck, Darmstadt, Germany) for 20 min at room temperature, dehydrated with 70 per cent ethanol/0.01 n HCl, treated with 1 m sodium thioceyanate (Merck) at 80°C for 10 min, and digested with 4 mg/ml pepsin from porcine stomach mucosa (2500-3500 units per mg protein; Sigma) in 0.02 n HCl at 37°C for 20 min. Additionally, slides were dehydrated in 70 per cent ethanol/0.01 n HCl and digested with 0.1 per cent Carlsberg protease (Sigma protease XXIV, Sigma) in 0.1 m Tris–HCl, pH 7.5 and 0.05 m NaCl for 10 min at 37°C and dehydrated again. The pretreated paraffin sections were denatured in a moist chamber for 5 min at 80°C. After overnight hybridization with the DNA probes (4 ng probe for chromosome 1 and 10 ng probe for the other chromosomes) at 37°C, sections were washed twice for 5 min in 2 × SSC/0.05 per cent Tween 20 buffer, pH 7.0 at 42°C and twice for 5 min in 4 × SSC/0.05 per cent Tween 20 buffer at room temperature. The ABC protocol (Vector) was applied for detection of the biotinylated probe in the procedure combining immunocytochemistry and in situ hybridization. Peroxidase activity was visualized by a diaminobenzidine (DAB; Sigma) precipitation reaction. Counterstaining was performed with haematoxylin and slides were dehydrated and mounted in Entellan (Merck).

Cytospins of the cell suspension from the fresh lymph node biopsy were denatured in a moist chamber for 5 min at 75°C, hybridized with directly fluorochrome labelled DNA probes overnight at 37°C, and washed twice for 5 min in 2 × SSC/0.05 per cent Tween 20 buffer, pH 7.0 at 42°C, twice for 5 min in 0.1 × SSC/0.05 per cent Tween 20 buffer at 60°C and finally once for 5 min in 4 × SSC/0.05 per cent Tween 20 buffer at room temperature. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma; 1:25 ng/ml) diluted in glycerol containing 2:3 per cent 1,4-diazobicyclo(2.2.2)-octane (DABCO; Sigma).

Combined immunocytochemistry and in situ hybridization on paraffin sections

A protocol was established for the combination of immunocytochemistry and in situ hybridization on paraffin sections for bright-field microscopic analysis. CD30 reactivity was detected with a TMB precipitate reaction and recorded with a CCD S30 camera (Meta-systems, Sandhausen, Germany) using the ISIS program of Metasystems (Sandhausen). X- and Y-coordinates of CD30-positive and CD30-negative cells were determined and accessory images of an England finder (Graticules Limited, Tonbridge, Kent, U.K.) were recorded. Subsequently, the precipitate was dissolved in phosphate-buffered saline (PBS), resulting in nuclei that were more accessible to the DNA probes used in the in situ hybridization protocol, which was applied as described above. The results of the in situ hybridization reaction were analysed and recorded after reallocation of the cells on the slide using the X- and Y-coordinates.
and the England finder images. The scoring criteria for the evaluation of the in situ hybridization results have been described earlier by Hopman et al.\textsuperscript{22,24} Finally, the results of CD30 immunocytochemistry and in situ hybridization were combined for the determination of the CD30 phenotype of the chromosomally abnormal cells.

**Cell type evaluation**

The pathologists independently evaluated the chromosomally abnormal cell types using CD19, CD20, and CD30 staining and cell morphology. Hodgkin cells were defined in this evaluation as large CD30-positive cells, with a large and distinct nucleolus. Reed–Sternberg cells, on the other hand, were defined as large CD30-positive bi- or multinucleated cells. Only areas with CD30-positive Hodgkin and Reed–Sternberg cells were evaluated in the sections. Morphologically normal cells in this paper are lymphocytes without characteristics of Hodgkin cells or Reed–Sternberg cells.

**RESULTS**

**Case 1**

Immunocytochemistry and in situ hybridization procedures were combined on a paraffin-embedded bone marrow biopsy in order to characterize the phenotype of chromosomally abnormal cells. Evaluation of the CD30 staining reaction in paraffin sections of this biopsy revealed that approximately 20 per cent of the cells in this preparation were CD30-positive Hodgkin and Reed–Sternberg cells, while the remaining compartment comprised CD30-negative bystander cells. About 300 cells could be evaluated for CD30 positivity or negativity in combination with their chromosome 1 copy numbers (Figs 1a–1d). In approximately 10 per cent of cells, numerical chromosome 1 abnormalities were detected. One-third of aneuploid nuclei clearly belonged to CD30-negative, morphologically normal cells. Trisomy for chromosome 1 was mainly observed, although multiple copies of chromosome 1 were also detected, but were only seen in morphologically abnormal Hodgkin/Reed–Sternberg cells. More copies may be present, but lost due to nuclear truncation in tissue sections. The largest proportion (approximately 66 per cent) of nuclei with an aberrant chromosome 1 copy number was observed in the CD30-positive Hodgkin and Reed–Sternberg cell population.

**Case 2**

The karyotype obtained from the fresh lymph node biopsy of this male patient revealed 10 per cent of 50 metaphase spreads with one extra copy of chromosome X, and no additional numerical or structural chromosome abnormalities. Peripheral blood lymphocytes showed a normal XY constitution in 100 analysed cells.

In situ hybridization for chromosomes X, Y, and 1 was combined with immunocytochemistry for CD30 and CD19 to determine the phenotypic constitution of the chromosomally abnormal cells. This was performed on 4 μm thick frozen sections and on a cell suspension obtained from the fresh lymph node biopsy (Fig. 1e–1m). Of the 1000 cells analysed, a minority showed two copies of chromosome X (3·5 per cent), loss of the Y chromosome (3·0 per cent), or an extra copy of chromosome 1 (2·2 per cent) (Fig. 1e–1g). Approximately half of the cells with an extra X chromosome were CD30-negative and appeared morphologically normal. These genetically aberrant cells expressed CD19 (Fig. 1k–1m), whereas Hodgkin/Reed–Sternberg cells lacked CD19 and CD20. Furthermore, loss of the Y chromosome was not limited to the CD30-positive Hodgkin/Reed–Sternberg cells. Again, approximately half of the cells genetically aberrant for the Y chromosome were CD30-negative, and appeared morphologically normal. In addition, approximately one-quarter of the cells with a gain in copy number for chromosome 1 were CD30-negative.

Multiple-target fluorescence in situ hybridization (Fig. 1h–1j) showed that half of the genetically abnormal, morphologically normal cells contained only a numerical abnormality for chromosome X (disomy), but not for chromosomes Y or 1, thus corresponding to the chromosomal constitution of the karyotyped cells. The other half of the genetically aberrant cells contained 50 per cent of cells with only a loss of the Y chromosome and 50 per cent of cells with disomy for chromosome X and no Y chromosome. CD30-negative morphologically normal cells with aberrant copy numbers of chromosome 1 were always seen with additional abnormalities of chromosomes X or Y. Finally, all Hodgkin/Reed–Sternberg cells contained an extra copy of the X chromosome, but had also lost the Y chromosome and contained an extra copy of chromosome 1 per nucleus.

Figure 2 provides a schematic overview of the correlation between the morphological, immunophenotypic, and genetic characteristics of the individual cell types present in this case.

**DISCUSSION**

In a recent study\textsuperscript{13} we observed that numerical chromosome abnormalities in Hodgkin’s disease are not restricted to Hodgkin/Reed–Sternberg cells, but are also detected in morphologically normal cells. The phenotypic constitution of these genetically aberrant, morphologically normal cells and their relationship to the Hodgkin/Reed–Sternberg cells could not, however, be investigated, due to their low frequency. In this study, tissue samples from two patients with classical Hodgkin’s disease containing a relatively large population of chromosomally abnormal cells were used to determine their nature.

Both cases showed numerical chromosome abnormalities in the CD30-positive Hodgkin/Reed–Sternberg cell population. However, CD30-negative, morphologically normal cells with aberrant chromosome copy
Fig. 1—Paraffin sections of a bone marrow biopsy from case 1 analysed for CD30 staining (a, c) and numerical aberrations for chromosome 1 with in situ hybridization (ISH) (b, d). It is demonstrated in corresponding areas of CD30 staining and ISH (a+b, c+d) that numerical chromosome aberrations are not restricted to the CD30-positive Hodgkin/Reed-Sternberg cells (white arrow-heads), but include also CD30-negative, morphologically normal cells (white arrows). Cell suspensions from the second patient analysed with the FICTION technique (e–g, k–m) or with multiple-target fluorescence in situ hybridization (h–j). CD30-positive Hodgkin/Reed-Sternberg cells (red: TRITC-positive) and CD30-negative morphologically normal cells (arrows) with numerical aberrations for chromosome X (one extra copy) (e), chromosome Y (loss) (f), and chromosome 1 (one extra copy) (g). Double-target FISH demonstrated numerical abnormalities for only chromosome X (two red TRITC signals) but not for the Y chromosome (one green FITC signal) in a morphologically normal cell (h). Chromosome abnormalities for X (two red TRITC signals) and Y (loss of green FITC signal) were also observed in a morphologically normal cell (i). Triple-target FISH showed numerical aberrations for chromosomes X (two red TRITC signals), Y (loss of green FITC signal), and 1 (three yellow TRITC/FITC signals) in a morphologically abnormal Hodgkin/Reed-Sternberg cell (j). CD19-positive, morphologically normal cells (red signal) with numerical aberrations for chromosome X (one extra green signal) in a B-cell-rich area (arrow) (k), and an area with a low frequency of B-lymphocytes (l, m).
numbers were also observed. To circumvent false-negative results due to CD30 staining heterogeneity, only areas with strong CD30 staining in the Hodgkin/Reed–Sternberg cells were evaluated. The morphology of genetically abnormal cells was also evaluated, showing that these cells did not have a Hodgkin/Reed–Sternberg cell morphology. These data together demonstrate that CD30-negative, genetically aberrant, but morphologically normal cells do exist in Hodgkin’s disease.

The phenotypic and genotypic constitution of the CD30-negative, morphologically normal cells and CD30-positive Hodgkin/Reed–Sternberg cells was further characterized in cell suspensions and frozen sections of the second case. The B-cell antigen CD19 was observed in genetically abnormal but morphologically normal cells. In contrast, the Hodgkin/Reed–Sternberg cells in our cases did not express CD19 in frozen sections. Furthermore, in paraaffin sections, Hodgkin/Reed–Sternberg cells in the analysed cases were negative for another pan B-cell marker, CD20, which is in accord with the reported literature, which demonstrates heterogeneous staining with B-cell markers.25,26 However, single cell analysis of Hodgkin/Reed–Sternberg cells showed that the typical immunoglobulin gene rearrangements could be detected, although B-cell antigen expression was lacking, demonstrating that they are derived from B-lymphocytes.11,30

The genotypic constitution was investigated in more detail in order to assess the relationship between the genetically abnormal but morphologically normal B-lymphocytes and the Hodgkin/Reed–Sternberg cells. The karyogram after 48 and 72 h culture revealed one extra copy for chromosome X, with no other numerical chromosome abnormalities. CD30 staining and single- and multiple-target in situ hybridization detected, in addition to the disomy for chromosome X, numerical abnormalities for chromosomes Y and 1 in CD30-positive Hodgkin/Reed–Sternberg cells. Such discordant results were previously also obtained by Weber-Matthiesen et al.31 Consequently, the karyotyped cells could not have originated from CD30-positive Hodgkin/Reed–Sternberg cells.

To detect the origin of the observed karyotype, CD30-negative, morphologically normal cells were also characterized. It was demonstrated that several populations existed of CD30-negative, morphologically normal cells with numerical abnormalities for chromosomes X, Y or 1. The largest fraction of genetically aberrant, morphologically normal cells had only one extra copy of chromosome X, with no additional abnormalities of chromosomes Y and 1. These cells were therefore designated as the source of the observed abnormal karyotype. Furthermore, they express CD19 and are clonogenic, based on the observation that this karyotype was obtained in several dividing cells. Hence, these genetically aberrant, morphologically normal B-lymphocytes might be progenitor cells of the malignant Hodgkin/Reed–Sternberg cells, because they are genetically related to each other; additional numerical chromosome abnormalities have, however, accumulated in the Hodgkin/Reed–Sternberg cell population.

A model for the development from progenitor cell to malignant cell can be described according to the relationships, mentioned above, between the genetically aberrant, morphologically normal cells and the malignant Hodgkin/Reed–Sternberg cells observed in the second case (Fig. 2). Phenotypically, the CD19 antigen is

Fig. 2—A model for tumour cell development in a male patient with nodular sclerosing Hodgkin’s disease proposed on the basis of the results of the karyotyping and the combined immunocytochemical and in situ hybridization technique. CD30-positive Hodgkin/Reed–Sternberg cells have numerical abnormalities for chromosomes X, Y, and 1, whereas karyotyped cells are only abnormal for chromosome X. Single-target in situ hybridization combined with CD30 staining clearly demonstrated that CD30-negative cells are numerically abnormal for chromosomes X, Y or 1. Multiple-target in situ hybridization showed that karyotyped cells originated from CD30-negative morphologically normal cells. Cells that lost the Y chromosome were not found with karyotyping, indicating that these cells have a growth disadvantage and therefore do not contribute to the developmental route.
lost during the transformation, whereas the CD30 antigen is acquired.\textsuperscript{32} It would be interesting to determine the differentiation status, i.e. lamin expression, of the morphologically normal cells, because we observed in a previous study that Hodgkin/Reed–Sternberg cells displayed a relatively differentiated phenotype according to their A-type lamin expression.\textsuperscript{33} Genetically, progenitor cells gain one extra copy of chromosome X, as seen in the karyotype, followed by loss of the Y chromosome and finally the acquisition of an extra copy of chromosome 1, resulting in Hodgkin/Reed–Sternberg cells which assembled numerical abnormalities for all three chromosomes. The population of cells with only loss of the Y chromosome was not observed in the karyotype, implying a growth disadvantage for this clone and suggesting that this fraction did not contribute to the follicle centre has still to be assessed.

In summary, immunocytochemistry combined with \textit{in situ} hybridization marked a population of CD30-negative, morphologically normal B-lymphocytes with numerical chromosome abnormalities. Since these cells show aneuploidy for certain chromosomes, they have been regarded as malignant cells. Furthermore, some of these cells proliferate \textit{in vitro}. Finally, these genetically abnormal but morphologically normal B-lymphocytes might be progenitors of the Hodgkin/Reed–Sternberg cells, due to their genetic relationships. However, definitive proof will only be obtained after determination of the immunoglobulin gene rearrangements and their mutations in the genetically abnormal, morphologically normal cells and Hodgkin/Reed–Sternberg cells.

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REFERENCES

3. Weber-Matthiesen K, Deerberg J, Schlegelberger B. Numerical chromosome aberrations are present within the CD30+ Hodgkin and Reed-Sternberg cells in 100% of analyzed cases of Hodgkin’s disease [see comments]. \textit{Blood} 1995; 86: 1464-1468.