Review

Single cell PCR for the analysis of Hodgkin's disease: Four years later

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Summary

Background: Single cell-based studies represent a promising alternative to conventional molecular approaches in the study of Hodgkin's disease since the malignant Hodgkin and Reed–Sternberg cells (H & RS) represent only a small minority of the cellular infiltrate in affected nodes.

Methods: Single cell polymerase chain reaction (PCR) assays were developed for the analysis of specific genomic DNA sequences and the detection of gene expression. Single H & RS cells were isolated by micromanipulation from cytospin slides or fresh cell suspensions after staining with an anti-CD 30 MoAB.

Results: The status of oncogenes and immune receptor genes was examined by DNA-PCR. So far, no IgH or TCR gamma rearrangements were detected in H & RS cells of T- and B-antigen negative classical Hodgkin's cases but were detected in two cases of nodular paragranuloma. Global cDNA amplification was successfully performed from single H & RS cells, and specific gene transcripts were detected with a novel PCR method.

Conclusion: Single cell PCR is a novel and promising method that will help to elucidate many of the open questions in the biology of Hodgkin's disease. In the case of contradictory results, collaborations between different groups utilizing similar approaches have to be performed.

Key words: cDNA libraries, clonal heterogeneity, Hodgkin's disease, immunoglobulin heavy chain rearrangements, polymerase chain reaction, single cell isolation

Background

Hodgkin and Reed–Sternberg (H & RS) cells are believed to represent the malignant cell population in Hodgkin's disease due to their ability to disseminate, their multinuclearity and the results of cytogenetic studies showing aneuploidy and clonality [1]. Due to their scarcity in affected tissues, studies aimed at the biologic and molecular characterization of H & RS cells have been difficult to perform and have yielded conflicting results. However, these conflicting results may also be due to the biological and clinical heterogeneity of the disease. Only studies at the level of a single H & RS cell will be able to solve the numerous open questions in Hodgkin's disease [2, 3]. Recent advances in molecular biology, especially the development of the polymerase chain reaction (PCR) by Mullis [4], enabled scientists to study molecular events at the single-cell level [5, 6]. Our group has applied these techniques to the study of Hodgkin's disease and presented the first results at the 2nd International Symposium on Hodgkin's disease [7]. Since then, numerous questions have been addressed with the novel techniques developed by our group [8]. This article summarizes the technical details for the analysis of genomic DNA and gene expression at the level of single H & RS cells.

Materials and methods

Isolation of H & RS cells for DNA and RNA analysis

Fresh lymph nodes from patients with Hodgkin's disease are dissected into small pieces with sterile needles. The H & RS cells are isolated by density gradient centrifugation (Ficoll Paque, Pharmacia, Freiburg, Germany) for 30 minutes together with the fraction of mononuclear cells. After fixation for 20 minutes in a solution of paraformaldehyde (3% w/v), the suspension is cytocentrifuged onto glass slides and stained for the presence of the CD 30 antigen with the alkaline-phosphatase-anti-alkaline-phosphatase-assay (APAAP) [9] using purified monoclonal antibody HRS-4. For RNA analysis, cell suspensions are washed after centrifugation, incubated with anti-CD30-antibody and resuspended in PBS. H & RS cells are identified according to morphological criteria like size, prominent nucleoli, multiple nuclei in the case of Reed–Sternberg cells as well as positive staining for the CD 30 antigen. Single cells are picked with glass capillaries using a micromanipulator (Eppendorf, Hamburg, Germany) coupled to a PixCell system (Stoelting, Chicago, U.S.A.), transferred into reaction tubes and digested with proteinase K in the case of DNA analysis.

PCR for the detection of IgH gene rearrangement

A mixture of six VH primers, two consensus JH primers in a final concentration of 15 nM each, 200 μM of each dNTP, 2 mM MgCl₂ and 1 U Taq DNA polymerase (Goldstar, Eurogentec, Belgium) in 30 μl 1 × PCR reaction buffer is added to the cells. The six different VH primers correspond to consensus sequences of the 6 different VH-families in the framework region 1 (V1–5'CAGTGAGG-GTG(T)TCCTGCAGGC3'; V2–5'CAGTGAGG-GTG(T)TCCTGCAGGC3'; V3–5'CAGTGAGG-GTG(T)TCCTGCAGGC3'; V4–5'CAGTGAGG-GTG(T)TCCTGCAGGC3'; V5–5'CAGTGAGG-GTG(T)TCCTGCAGGC3'; V6–5'CAGTGAGG-GTG(T)TCCTGCAGGC3').
G3'; V4-5'CCTGTCCCTCACCTGC(AG)CTGTC3'; V5-5'AAA-
AAGCCCGGGAGTCTCTGA(AG)GA3'; V6-5'CTGTGCCAT-
CTCCGGGGACAGTG3'). The sequences of the JH primers used,
one as consensus sequence for the segments J1, 2, 4, 5 (5'GACT-
CACCTGAGGAGACGGTGACC3) and one for the segments J3
and J6 (5'ACTTACCTGAGGAGACGGTGACC3) were deter-
mained according to the data published by Ravetch et al. [10]. The
samples are subjected to 40 temperature cycles (95° 90 seconds, 65”
30 seconds, 72° 80 seconds) using a thermal cycler (Biometra, Göt-
tingen, Germany). In a second PCR amplification, 1 μl of each
sample was added to each of a set of six vials, containing only one
of the VH primers each, and six nested JH primers (J1-5'CGTCGTC-
GAGGGTGCCCTGGCCCCAGTGC3'; J2-5'CGTCGTCGAC-
AGGGTGCCAGCCCCAGTG3'; J3-5'CGTCGTCGACATT-
GTCCCTTGGCCCCAGACATAC3'; J4-5'CGTCGTCAACCC-
GGTTCCTTGGCCCCAGTAG3'; J5-5'CGTCGTCGACGTGG-
TCCCTTGCCCCCAGG3'; J6-5'CGTCGTCGACGTGG-
TCCCTTGCCCCCAGTG3') in a final concentration of 125
nM. The JH primers carry a restriction site printed in bold charac-
ters. All experiments are performed using a reaction tube without
DNA as a negative control and tubes containing single cells from
various cell lines derived from Burkitt's lymphoma (B61P27, K601,
Raji, BL29, CA46) carrying different rearrangements as well as cells
from the peripheral blood of patients with CLL (JU, MB, KB;
Figure 2). All products are sequenced using a direct sequencing
protocol (Cycle Sequencing, Biozym Germany) to prove speci-
ficity.

Single-cell-cDNA-synthesis

cDNA synthesis was performed as described [5, 8, 11]. Briefly,
H & RS cells are dropped into an Eppendorf tube containing 4 μl of
1st Comp buffer (NP-40 25%, 10 μmol dNTPs, 10 nmol dt18-
primer, RNAse-inhibitors), reverse transcriptase (Gibco, Karlsruhe,
Germany) is added and a polyA-tail is subsequently added to the
cDNAs with terminal transferase (Boehringer Mannheim, Mann-
heim, Germany). The tailed cDNAs are amplified by PCR with a
single primer (NotI-dT18-primer [18]; Figure 3). The amplification
is carried out for 50 cycles, and products are separated on an
agarose gel resulting in a diffuse smear of 300–800 bp length. The
detection of specific cDNAs within the global polyA-PCR is pos-
sible by a second sequence-specific amplification with primers close
to the 3’-region of the genes of interest fi-actin and restin-primers
result in amplification products of 239 and 269 bp, respectively
(Figure 4). Representative PCR products are cloned and sequenced
to prove specificity.

Results

DNA-PCR

We have extended our previous analysis of immuno-
globulin heavy chain (IgH) rearrangements in single
H & RS cells [12] to a new series of 10 cases of
Hodgkin’s disease was excluded. Lymphocyte-predominant histology
was excluded from this analysis. None of these cases
showed expression of T- or B-cell associated antigens.
Cells from cell lines and CLL with rearrangements of
all known variable heavy chain (VH) families were
included a positive controls (Figure 2). The amplifica-
tion efficiency was approx. 70%, i.e., on average 7 out
of 10 cells gave bands of the appropriate size after
seminested PCR-amplification. Representative bands
were excised and sequenced to prove specificity and
exclude contamination. Rearrangements were not
detected in any of the single H & RS cells examined,
confirming our previous observation that these rear-
rangements are rare events in T- and B-antigen negative
classical Hodgkin’s disease. We have previously shown
that T-cell receptor gamma gene rearrangements do
not occur in single H & RS cells from the same cases
[13]. IgH rearrangements were detected in two cases of
classical nodular paragranuloma.

Analysis of EBV-specific sequences in single
H & RS cells [14] had previously confirmed the speci-
ficity of our selection approach since all H & RS cells
from EBV positive cases gave positive PCR results,
whereas lymphocytes from the same cases as well as
H & RS cells from EBV negative cases never gave
positive signals. N-ras mutations for codons 12/13 and

Figure 1. Isolation of a single H & RS cell by micromanipulation. A
single Hodgkin cell is shown at the tip of the glass capillary after
being lifted off the glass slide. The whole nucleus is transferred into
an Eppendorf tube whereas the cytoplasmic rim as well as surrounding lymphocytes remain on the glass slide.

Figure 2. Results of single cell IgH-PCR in cells of different B-cell
lines and single cells of CLL (chronic lymphocytic leukemia). IgH
sequences were amplified with a seminested PCR-approach from
single cells of different cell lines and single cells of B-cells from
patients with CLL. In every case, a strong band is seen at 350 bp.
These bands were excised and sequenced. Unique sequences com-
mon to all cells of a given cell line or case were obtained by sequenc-
ing several PCR products in each case.
suitable DNA probes etc. After successful global cDNA amplification, a broad smear is visible on the agarose gel (Figure 4, upper lane). Aliquots are then amplified with primers for β-actin (Figure 4, middle lane) to prove successful amplification and for the H & RS-cell specific gene restin [21]; (Figure 4, lower lane) to prove specificity with regard to the cell selection. This novel technique will enable us to examine the expression of interleukin and interleukin-receptor genes in a panel of single H & RS cells from different cases.

Summary

We have developed single cell PCR assays for the detection of specific genomic DNA sequences, e.g., oncogenes and immune receptor genes, as well as gene expression in single tumour cells of Hodgkin's disease. These studies were initiated to gather information on the molecular events underlying this still enigmatic disease. The cell selection process has been optimized with regard to the staining of H & RS cells. EBV-studies have proven the specificity of the H & RS cell selection. The information derived from gene expression studies revealed pronounced molecular heterogeneity between different cases as well as significant fluctuations in gene expression from cells of the same cases [11]. These studies will be extended to the analysis of cytokine gene expression in single H & RS cells. Whereas we did not find rearrangements of the IgH genes in single H & RS cells [12], groups using cells isolated from sections [22, 23] or suspensions prepared from paraffin-embedded sections [24] reported on monoclonal and polyclonal rearrangements in single H & RS cells. These conflicting results may either be due to technical factors inherent to the cell selection process and/or the PCR-system or may be due to biological differences between the cases chosen for analysis. We therefore propose the exchange of samples between the different groups currently working in the field. Despite these controversies on the topic of IgH rearrangement the technique of single cell PCR itself that was first presented at the last Cologne Symposium in 1991 [18] has emerged into one of the most promising technical advances in the study of Hodgkin's disease.

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Figure 4. Results of single cell polyA-PCR (global PCR) and specific amplification of β-actin and restin sequences. cDNA from 19 different H & RS cells was amplified by polyA-PCR (lane 1) and subsequently amplified by specific PCR for β-actin (239 bp band) or restin (269 bp band). PolyA-PCR generates a smear between 300 and 800 bp size that corresponds to a mixture of different cDNAs present in the original sample.

References

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