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Short Communication

The relevance of RT-PCR detection of disseminated tumour cells is hampered by the expression of markers regarded as tumour-specific in activated lymphocytes

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ABSTRACT

Marker genes, commonly used to detect circulating tumour cells in RT-PCR-based tests: squamous-cell carcinoma antigen, epidermal growth factor receptor, mammaglobin, small breast epithelial mucin, but not carbonic anhydrase 9, were shown to be expressed in normal, mitogen-stimulated peripheral blood mononuclear cells (PBMNC). Thus, considering the inflammatory reactions often accompanying cancer development, to reduce false-positive results of the metastatic tumour cell tests, molecular markers should be validated not against normal peripheral blood, but against activated lymphoid cells, such as *in vitro* mitogen-stimulated PBMNC.

1. Introduction

The early detection of disseminated disease in asymptomatic cancer patients may help to predict recurrences and to select more effective treatment regimens. To detect circulating tumour cells (CTC) in cancer patients, high sensitivity reverse transcription-polymerase chain reaction (RT-PCR)-based assays have been developed.[1] However, molecular markers

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used in these assays, the so-called cancer cell markers, present controversial specificity, and false-positive findings of RT-PCR assays are frequent. In effect, the last decade's constant attempts to apply RT-PCR for micrometastasis detection have so far failed to produce a commonly accepted, routinely applied diagnostic method.

The difficulties in developing reliable CTC detection tests may lie in the common, but mistaken assumption that markers of high specificity for CTC are those not detected in normal blood, and thus their specificity is evaluated against their expression in the peripheral blood of healthy volunteers. Yet accumulating evidence indicates that normal tissue is not an adequate control for assessing the specificity of molecular markers used for CTC detection. Already the early stage cancer was shown to affect gene expression patterns in peripheral blood cells.[2] The symptoms of ongoing inflammatory reactions, such as raised erythrocyte sedimentation rate, elevated concentrations of circulating C-reactive protein, high levels of numerous cytokines and increased percentages of activated T lymphocytes (e.g. CD4+HLA-DR+) are often exhibited by the peripheral blood of cancer patients.[3–9] Many of those parameters correlate with disease progression and prognosis,[3–5,7,8] and the systemic inflammatory response has been shown to be a strong independent prognostic factor in patients with advanced cancer.[10,11]

Considering the above, activated cells are the likely component of the peripheral blood, bone marrow or regional lymph nodes of cancer patients.

Therefore, to verify the specificity of cancer cell detection, we addressed the question as to whether some commonly used molecular markers are inducible in normal peripheral blood mononuclear cells (PBMNC). The following markers were selected for RT-PCR analysis: squamous-cell carcinoma antigen (*SCCA*), epidermal growth factor receptor (*EGFR*), mammaglobin (*hMAM*), small breast epithelial mucin (*SBEM*) and carbonic anhydrase 9 (*CA9*).

2. Materials and methods

2.1. Isolation and culture of PBMC and spleen lymphocytes

PBMNC from three healthy donors (samples 1, 2, 3) were obtained by standard Ficoll-Uropoline gradient centrifugation. For activation, PBMNC were cultured for 12 h and for 6 days in RPMI-1640 medium with L-glutamine (Gibco), supplemented with 10% heat-inactivated FCS (Gibco), and 50 μg/ml of gentamycin (Sigma), with the addition of phytohemagglutinine (PHA 1 μg/ml, Wellcome).

Spleen cells of three patients with immune thrombocytopenic purpura, obtained by mechanical dissection of spleens after therapeutic splenectomy, were centrifuged on Ficoll-Uropoline gradient. Mononuclear cells were cultured for 2 days in RPMI-1640 medium containing L-glutamine (Gibco), supplemented with 50 μg/ml of gentamycin and with human (to avoid stimulation by xenogenic antigens) heat-inactivated AB serum (10%). Following the culture, lymphocyte enrichment was performed by Nycodenz (Nycomed) gradient centrifugation.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was isolated from approximately 5×10^7 cells of each sample according to Chomczynski and Sacchi.[12] Two μg of the total RNA were reverse transcribed with random hexamer primers, in the final volume of 20 μl, using SuperScript (Invitrogen), according to the manufacturer's instructions. The quality of cDNA preparations was controlled by the PCR of DNA polymerase β (*pol* β) that is a single copy gene (containing no known pseudogenes) expressed in all cell types. Its transcript is rather stable in the cell cycle, and characterised by low-level expression, in contrast to house-keeping gene products commonly used as cDNA controls, such as β-actin and GAPDH. PCR for *pol* β was performed with 0.2 μl of cDNA as follows: 40 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, preceded by 4 min at 95 °C and followed by 7 min at 72 °C. Specific oligonucleotide primers, sense GATATTTTGGGGACTTTGAAA and antisense CTCTTCTGAAACTGCCACAG produced 129bp DNA fragment. Subsequently, 2 μl of cDNA was examined for *SCCA*, *EGFR*, *hMAM*, *SBEM* and *CA9* markers by PCR. Specific primers and PCR conditions used were described previously.[13–17] PCR was performed in a 25 μl mix using *Taq* Fast Start polymerase (Roche Diagnostics GmbH). The mixture (0.8 μl) after the first round of PCR served as a template for the second round of PCR. For each PCR, a set of control samples included one positive control, and two negative controls (a sample with no template and a sample with the placental DNA). As positive RT-PCR controls, the following human cell lines were used: MCF7 for *SCCA*, MCF7 and HeLa for *EGFR*; MCF7, HeLa and A431 for *hMAM*; MCF7 for *SBEM*; and A431 and HeLa for *CA9*.

3. Results and discussion

All the control cDNAs obtained from cell lines were RT-PCR positive for the respective markers. PCR controls with the placental DNA template were negative for all markers tested. The latter control excluded the putative pseudogene detection.

The representative results are shown in Fig. 1. We have demonstrated here that while normal PBMNC (donors 1 and 2) were RT-PCR negative for *SCCA*, *EGFR*, *hMAM*, *SBEM* and *CA9*, PHA-stimulated PBMNC, in both early (12 h following stimulation, samples 1S and 2S) and late (6 days following stimulation, sample 3S) phase of stimulation, were positive for *SCCA*, *EGFR*, *hMAM*, and *SBEM*, but not for *CA9*. This implies that any condition involving activated cells may contribute to false-positive RT-PCR results. In this respect, of the markers examined, *CA9* seems to present potentially the best value for RT-PCR-based CTC detection. However, because of its trace expression in sample 1, its activation-related transcription requires further investigation.

In addition, unstimulated, pooled lymphocytes obtained from spleens of three patients with immune thrombocytopenic purpura expressed *SCCA*, *EGFR* and *SBEM* but not *hMAM* and *CA9* transcripts (Fig. 1).

While the number of publications on the molecular tumour markers continues to grow, our results support and