Fetal cells in the peripheral blood of pregnant women express thymidine kinase: a new marker for detection

Markus Hengstschläger*, Gerhard Bernaschek

Obstetrics and Gynecology, Department of Prenatal Diagnosis and Therapy, University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria

Received 17 December 1996; revised version received 30 January 1997

Abstract Fetal cells occur in maternal blood in a substantial proportion of normal pregnancies. Several different approaches have been used to detect and enrich these cells for non-invasive prenatal diagnosis. However, before these fetal cells can routinely be used for prenatal diagnosis, perfectly reproducible procedures for detection and enrichment need to be established. We found that these fetal cells express high intracellular levels of the DNA precursor pathway enzyme thymidine kinase. Since normal adult peripheral blood cells do not exhibit any thymidine kinase activity, this enzyme is a potent new marker to detect and enrich fetal cells from maternal blood. We further describe the first successful application of a cytofluorometric thymidine kinase assay to detect fetal cells in the maternal circulation by virtue of their high thymidine kinase activity.

Key words: Prenatal diagnosis; Non-invasive; Thymidine kinase; Flow cytometer

1. Introduction

Prenatal diagnosis is based on non-invasive techniques, such as ultrasound or maternal serum screening. For more detailed information invasive procedures are used to remove fetal tissue, which then can be analysed for chromosome anomalies, molecular genetic diseases or biochemical variations. All currently available methods for fetal tissue sampling, such as amniocentesis, chorionic villus sampling or isolation of fetal umbilical cord blood, carry procedure-related risks for the fetus. Since the first detection of fetal cells in the maternal circulation [1], a series of methods have been applied in attempts to detect and enrich them for use in non-invasive prenatal diagnosis [2–7]. Circulating cells of fetal origin include trophoblasts, fetal lymphocytes, nucleated red blood cells and hematopoetic stem cells [3]. Most of the investigations to quantify the circulating fetal cells used Y-chromosome sequence analysis in pregnancies with male fetuses. Different approaches for quantitative DNA analysis revealed that in first- and second-trimester pregnancies the fetal/maternal cell ratio varies between 1:475 000 and 1:1 600 000. Thus in any 10 ml blood sample between 200 and 60 fetal cells are present [8,9]. Each fetal cell type has already been investigated with respect to its enrichability from maternal circulation. The results thus obtained are very promising and indicate that non-invasive prenatal diagnosis via these cells is probably within reach. However, it is without doubt that detection and enrichment techniques will have to be improved before these fetal cells can be routinely used for prenatal diagnosis. The major limiting factor is to find a very sensitive marker which is highly specific for fetal cells among the excess of adult cells.

We report that fetal cells in the maternal circulation express high levels of the DNA precursor pathway enzyme thymidine kinase (TK). Since normal peripheral adult blood does not exhibit any TK activity, we show this activity to be useful as a specific marker for fetal cells. Using this marker we were able to cytofluorometrically detect fetal cells in the excess of maternal cells. Our results open a new and potent possibility to detect and enrich fetal cells from the maternal circulation.

2. Materials and methods

2.1. Cells

Samples from patients at different weeks of pregnancy undergoing amniocentesis, chorionic villus sampling, or isolation of fetal umbilical cord blood for standard obstetric indications were used for analysis. These cells were directly analysed without prior cultivation. Nucleated cells from peripheral blood samples (20 ml, collected in heparin) were obtained by densitiy gradient centrifugation on Ficoll-Paque. The blood was mixed with phosphate-buffered saline (PBS) in a tube at the bottom of which Ficoll-Paque was gently layered. After centrifugation the cells at the interface between the plasma and red cells were collected and analysed. Permission to use parts of the obtained materials for the presented study was obtained from the institutional review board. Fetal and adult fibroblasts were obtained from the American Type Culture Collection.

2.2. Radioactive thymidine kinase assay in cell extracts

Thymidine kinase activity in vitro was measured by conversion of radioactive thymidine to thymidine monophosphate [10]. Enzyme activities were normalized to total protein concentration, determined using the BioRad protein assay reagent with bovine serum albumin (BSA) as a standard.

2.3. Cytofluorometric thymidine kinase assay

Synthesis and purification of the fluorescent thymidine analogue AUdR/DANS were published previously, and cytofluorometric measurement of TK activity in living cells was performed as described [11,12]. Cells were exposed in RPMI medium without serum for 30 min to 1.5 mM AUdR/DANS (a stock solution was prepared in 70% ethanol) at 37°C and 7.5% CO_2. After harvesting by centrifugation for 5 min at 1000 rpm, cells were washed twice with PBS. The cells were resuspended in 100 mM Tris-HCl (pH 7.4), 154 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% NP-40 and 0.2% BSA (a 10× stock solution of BSA was stored at −20°C and it was thawed briefly before analysis) to a concentration of about 1×10^6 cells/ml. DNA staining was performed with 1.5 μg/ml ethidium bromide for 10 min on ice. Cells were analysed within the following 20 min to ensure that they were still alive and that the intracellular level of phosphorylated thyimidine analogue remained unchanged. TK activity and DNA content were simultaneously measured with a Partec PAS-II flow cytometer. Filters were set to obtain a complete separation of emission of the fluorescent analogue (500 nm) and of ethidium bromide (605 nm).

2.4. Single-cell isolation and PCR analyses

For single-cell isolation [13] cells were washed and diluted with

*Corresponding author. Fax: (43) 1-40400-7848.
E-mail: Markus.Hengstschlaeger@akh-wien.ac.at

PII S0014-5793(97)00144-0

PBS. The cell supension was spotted in microdrops (0.1–0.2 μl) onto cleaned coverslips, that were previously coated with 0.1 μl of endonuclease-free BSA (0.1 mg/ml in 0.1×PBS) to prevent cell adherence to the coverslip. The albumin-containing microdrops were allowed to dry before application of the cells. TK activity of the separated cells was determined under the microscope after incubation with the fluorescent thymidine analogue AUdR/DANS as described in [14]. Microdrops containing only one live cell were flushed with 1 μl of PBS and pipetted into a microreaction tube containing 3.5 μl of lysis buffer. Microdrops containing no visible cells were picked as negative controls. Lysis buffer was 0.68% Nonidet P-40, 0.68% Tween-20, 26.2 mM Tris-HCl (pH 8.3), 75 mM KCl, 2.2 mM $MgCl_2$ and 57 μg/ml proteinase K. The lysates were covered with a drop of mineral oil and incubated at 52°C for 30 min. Proteinase K was then heat-inactivated by incubating the tubes for 20 min at 95°C. After heat inactivation first-round PCR amplifications were performed in 10 μl reactions containing 5 pmol of each primer, and 0.5 mM of each deoxynucleoside triphosphate in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.005% (w/v) gelatin and 1 U of Taq polymerase.

Y-chromosome-specific PCR was performed by a dual amplification procedure using the primers Y1.5 (5′-CTAGACCGCAGAGGCGCCAT-3′) and Y1.6 (5′-TAGTACCCACGCCTGCTCCGG-3′) in a first-round PCR. One microliter of the first-round product was reamplified by nested PCR with Y1.7 and Y1.8 (5′-CATCCAGAGCGTCCCTGGCTT-3′ and 5′-CTTTCCACAGCCACATTTGTC-3′). Each round consisted of 30 cycles of 94°C for 1 min, 60°C for 30 s and 72°C for 2 min. We used two primers specific for sequences of the *HER-3* oncogene as internal control (5′-GACAAACACTGGTGCTGATC-3′ and 5′-GCCCTTTTATTCTGAATCCG-3′, compare [15]).

3. Results

We have analysed different fetal and adult cell types for intracellular TK activity using a radioactive enzyme assay. Trophoblasts, amniotic cells, fetal and adult fibroblasts as well as fetal umbilical cord blood cells expressed TK enzyme activity, whereas adult peripheral blood did not exhibit measurable activity (Fig. 1). Since TK activity is known to be S-phase regulated (see e.g. [10]), we wondered whether these differences might be determined by different proliferation conditions of these cells. However, flowcytometric analysis revealed that the trophoblast samples, the amniotic cells, the fetal cord blood and the adult peripheral cell populations all contained between 90 and 95% G0 or G1 cells with 2n DNA content (see Fig. 2). The fibroblasts exhibited a DNA distribution typical for logarithmically growing cells (data not shown). Here it is important to note that the amniotic cells were analysed directly after the sampling procedure and not after growth cultivation. From chromosomal analyses we further knew that the analysed trophoblast samples contained dividing cells. Therefore, the observed DNA distributions indicate that only a few cells in the samples still divide or that the cells proliferate very slowly, reflected by a very long G1 phase. We confirmed the obtained data on DNA distributions by analysing the incorporation of radioactive thymidine into DNA. We did not observe significant levels of thymidine incorporation in any of these cells, except fibroblasts (data not shown). However, we could not really clarify whether the fetal cells express TK activity because they still divide very slowly or because TK activity is aberrantly expressed in the Go/G1-phase of the cell cycle.

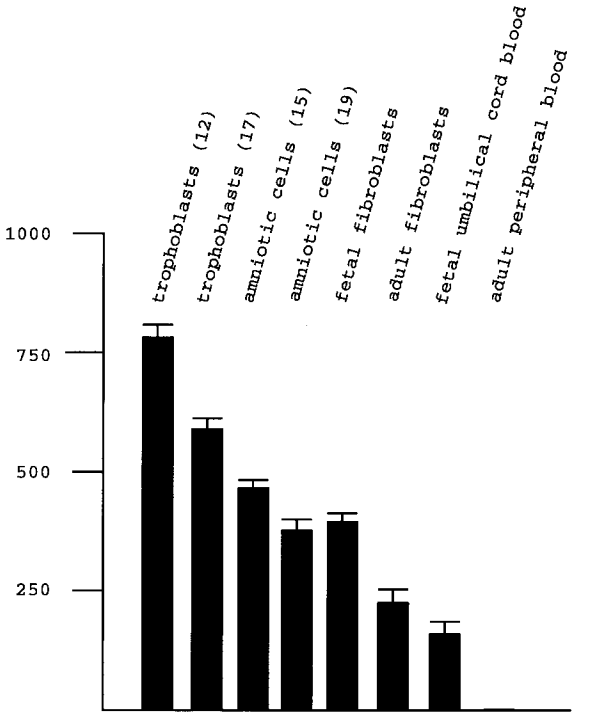

Fig. 1. TK activity in different cell extracts. Protein was extracted from different primary human cells and analysed for intracellular TK activity via the traditional radioactive TK assay. TK activity is given in picomoles TMP formed per miligram protein per hour. Results of three independent determinations are presented. The numbers given in brackets reflect the week of pregnancy at which the sample was isolated.

To further investigate this issue we performed simultaneous measurements of TK activity and DNA content of theses cells. We used a cytofluorometric assay, recently established in our laboratory, to detect transformed cells with high TK activity in an excess of normal cells with low activity

Table 1
TK-positive cells in the maternal circulation contain Y-chromosome DNA

Patient	Week of pregnancy	FACS calculation of TK+ in TK− cells[a]	Single cell PCR[b]	
			TK+/Y+	TK−/Y+
1	10	1:750 000	2/2	6/0
2	11	1:560 000	2/2	5/0
3	12	1:820 000	1/1	8/0
4	12	1:980 000	2/2	4/0
5	13	1:880 000	2/2	8/0
6	15	1:790 000	3/3	7/0
7	19	1:590 000	3/3	8/0

[a]The ratio of TK-positive cells (TK+) in TK-negative cells (TK−) in maternal peripheral blood was estimated on the flowcytometer as shown in Fig. 2.
[b]Single cell PCR was performed as described in Section 2. TK+ and TK− cells isolated by a minimal dilution procedure were tested for Y-chromosome DNA. Only those experiments with positive PCR signals using the control oligonucleotides for the oncogene *HER-3* are presented.

[11,12,16,17]: The cells are incubated with the fluorescent thymidine analogue AUdR/DANS. Intracellular accumulation of this analogue reflects TK activity. The DNA of these cells is further stained with ethidium bromide. These two fluorescences are simultaneously detected on a flow cytometer (for details of the method see [11], and Section 2). By this method we detected TK activity in trophoblasts, amniotic cells and fetal cord blood cells (Fig. 2). Analysing peripheral blood cells (about 30 million per individual) of 24 non-pregnant women (without any previous pregnancy) and 13 male individuals, we could not detect TK-positive cells (Fig. 2, see also [17]). Strikingly, using the same approach to analyse 14 pregnant women we observed a very small cell population with high TK activity (Fig. 2). For these analyses we chose women between 10 and 21 weeks of pregnancy without any previous pregnancies. We did not observe significant qualitative or quantitative differences in the cytofluorometric detection of TK-positive cells in the patients at different stages of pregnancy (see some examples in Table 1). We estimated the cell numbers of these small TK-positive populations to be between 30 and 60 in 30 million of total analysed cells.

To prove that these cells with high TK activity, which we specifically detected in the circulation of pregnant women, were indeed of fetal origin, we chose the following experimental approach. We isolated nucleated cells from 20 ml of peripheral blood of a woman pregnant with a male fetus. We incubated these cells in a reaction tube with the fluorescent thymidine analogue AUdR/DANS as described for the cytofluorometric TK assay in Section 2. Next we divided these cells into smaller populations of about 100 cells and microscopically analysed them for TK expression. Cell populations which contained one TK-positive cell, such as cell 1 in Fig. 3A, were designated as cell type 1. Populations with no detectable TK-positive cells were designated as cell type 2. We extracted DNA and analysed using PCR for the presence of Y-chromosome sequences. As an internal control of the experimental procedure we co-amplified sequences specific for the oncogene *HER-3*. All 10 cell populations of type 1 thus obtained contained Y-chromosome DNA, whereas none of the 74 analysed type 2 cell populations contained any Y-chromosome sequences (Fig. 3B).

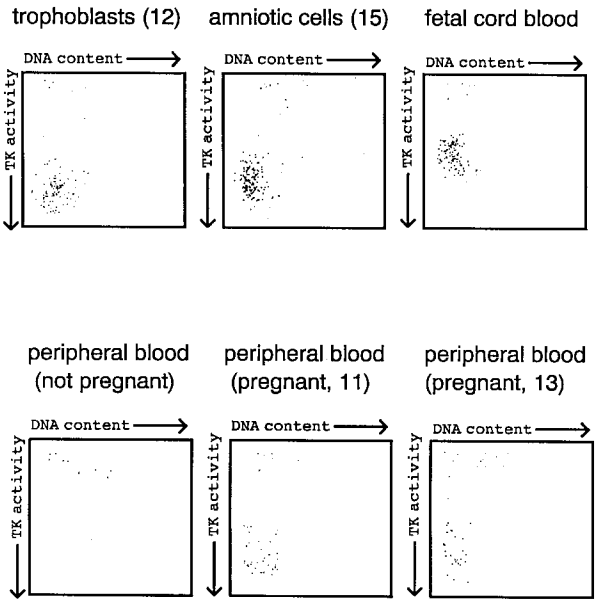

Fig. 2. Simultaneous cytofluorometric detection of DNA content and TK activity in living cells. About 8000 primary trophoblasts, amniotic cells, or fetal cord blood cells were cytofluorometrically analysed as described in Section 2. In case of adult peripheral blood of non-pregnant and pregnant women about 30 million cells were analysed. A two-dimensional presentation of DNA content (abcissa) against TK activity (ordinate) is shown (not that increasing enzyme activity goes down the axis!). The numbers given in brackets reflect the week of pregnancy at which the sample was isolated.

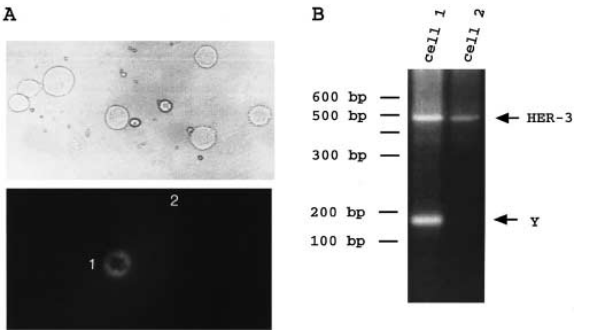

Fig. 3. Those cells in the maternal circulation with high TK activity are of fetal origin. A: Peripheral blood cells of a woman pregnant (11th week) with a male fetus were incubated with the fluorescent thymidine analogue AUdR/DANS and microscopically analysed for TK expression. Accumulation of the fluorescent analogue reflects intracellular TK activity. B: DNA of a maternal peripheral blood cell population containing a TK-positive cell (cell 1) and of a population not containing any TK-positive cells (cell 2) was extracted. The extracted DNA was analysed by PCR for Y-chromosome sequences and, as a control, for the *HER-3* oncogene.

One might imagine that something special about pregnancy could cause the appearance of TK-positive adult cells only in pregnant women. Accordingly, we next wanted to exclude the possibility that some of the detected TK-positive cells are of maternal origin. Nucleated blood cells of seven women at different stages of pregnancy were incubated with AUdR/DANS and microscopically analysed for TK expression (as shown in Fig. 3). We isolated single TK-positive and TK-negative cells by a minimal dilution procedure described in Section 2. Single-cell PCR analyses were performed to detect Y-chromosome sequences and the fetal sex was later confirmed by ultrasound. As shown in Table 1 we never detected Y-sequences in 46 TK-negative cells, whereas all 15 TK-positive cells analysed contained Y-chromosome DNA. We concluded that the Y-chromosome DNA was specific for the TK-positive cells, which proves them to be of fetal origin.

4. Discussion

The discovery of fetal cells in the circulation of pregnant women has been a stimulus for the development of technologies to detect and enrich these cells in order to use them for non(less)-invasive prenatal diagnosis. However, despite some initial promising results, the reproducibility and reliability of these techniques are still limited, mainly due to the lack of very specific markers for fetal cells [2–7]. We describe TK activity to be a new specific marker for fetal cells in the maternal circulation. We further report the successful application of a recently established cytofluorometric TK assay to visualize a very small cell population of high TK activity in the excess of TK-negative adult cells. These TK-positive cells in the maternal blood can be concluded to be of fetal origin for

the following reasons. (1) It has been demonstrated that circulating cells of fetal origin include trophoblasts, fetal lymphocytes, nucleated red blood cells and hematopoetic stem cells [3]. We showed that trophoblasts and fetal cord blood cells express high intracellular levels of TK activity, whereas normal adult peripheral blood is totally TK-negative (this study and [17]). (2) The small cell population with high TK activity specifically occurs in peripheral blood samples of pregnant women and not in the circulation of non-pregnant individuals. (3) The observed ratio of TK-positive cells in the blood of pregnant women (between 1:500 000 and 1:1 000 000) is very comparable to the earlier reported fetal/maternal cell ratio (between 1:475 000 and 1:1 600 000), estimated by other methods [8,9]. (4) By single-cell PCR analyses we demonstrated that TK-positive cells in blood of women pregnant with a male fetus contain Y-chromosome DNA, whereas TK-negative blood cells do not. We did not observe higher success rates of the single cell PCR in fetal male cells than in maternal female cells as shown by co-amplification with control oligonucleotides specific for the oncogene *HER-3*.

One question still remained. Do fetal cells in the maternal circulation express the normally S-phase regulated TK activity because they still divide or do they exhibit deregulated TK? We favour the latter explanation, since it has earlier been shown that fetal cells in maternal blood are not responsive to mitogens [18]. Proliferation of fetal cells in maternal blood is probably down-regulated because these additional cell divisions would seem disadvantageous for these cells and for the maternal circulation. In addition, from preliminary data showing that fetal cells exhibit a totally deregulated TK activity (G. Graf, M. Hengstschläger and E. Wawra, unpublished observation), one could imagine these circulating fetal cells to express TK activity independently from S-phase.

Experiments to enrich fetal cells by fluorescence-activated cell sorting with the marker described here are planned in our laboratory. Our data provide evidence that fetal cells express TK activity constitutively throughout much of the pregnancy. However, detailed analyses will have to be performed to test whether there are peaks of TK expression, which would be perfectly suited for routine application of this approach.

Acknowledgements: Work in M.H.'s laboratory is supported by Grant P10833-MED from the 'Fonds zur Förderung der wissenschaftlichen Forschung' and by the 'Komission Onkologie' of the Medical Faculty of the University of Vienna and by the Austiran 'Nationalbank'. The authors wish to thank Elke Hengstschläger-Ottnad, Gabriele Hölzl, Oliver Pusch, Thomas Soucek and Edgar Wawra for helpful discussion and critically reading the manuscript.

References

[1] Schmorl, G. (1893) Pathologisch-anatomische Untersuchungen über Puerperal-Eklampsie, Vogel, Leipzig.
[2] Adinolfi, M. (1995) Prenat. Diagnosis 15, 889-896.
[3] Gänshirt, D., Garritsen, H.S.P. and Holzgreve, W. (1995) Curr. Opin. Obstet. Gyn. 7, 103-108.
[4] Elias, S. and Simpson, J.L. (1993) in Essentials of Prenatal Diagnosis, pp. 381-389, Churchill Livingstone, 1993.
[5] Bianchi, D.W., Klinger, K.W., Vadnais, T.J., Demaria, M.A., Shuber, A.P., Skoletsky, J., Midura, P., Diriso, M., Pelletier, C., Genova, M., Erikson, M.S. and Williams. J.M. (1996) Prenat. Diagnosis 16, 289-298.
[6] Bianchi, D.W. (1995) J. Pediatr. 127, 847-856.
[7] Simpson, J.L., Lewis, D.E., Bischoff, F.Z. and Elias, S. (1995) Prenat. Diagnosis 15, 907-912.
[8] Hall, J.M., Adams, S., Williams, S., Rehse, M.A., Layton, T.J. and Molesh, D.A. (1994) Ann. NY Acad. Sci. 731, 115-127.
[9] Bianchi, D.W. (1994) Ann. NY Acad. Sci. 731, 92-102.
[10] Hengstschläger, M., Knöfler, M., Müllner, E.W., Ogris, E., Wintersberger, E. and Wawra, E. (1994) J. Biol. Chem. 269, 13836-13842.
[11] Hengstschläger, M. and Wawra, E. (1993) Cytometry 14, 39-45.
[12] Hengstschläger, M. and Wawra, E. (1993) Br. J. Cancer 67, 1022-1025.
[13] Liu, A.H., Creadon, G. and Wysocki, L.J. (1992) Proc. Natl. Acad. Sci. USA 89, 7610-7614.
[14] Hengstschläger, M., Hengstschläger-Ottnad, E., Pusch, O. and Wawra, E. (1996) Oncogene 12, 1635-1643.
[15] Hengstschläger, M., Hölzl, G., Ulm, B. and Bernaschek, G. (1997) J. Med. Genet. in press
[16] Hengstschläger, M., Pusch, O., Hengstschläger-Ottnad, E., Ambros, P.F., Bernaschek, G. and Wawra E. (1996) DNA Cell Biol. 15, 41-51.
[17] Hengstschläger, M., Pfeilstöcker, M. and Wawra, E. (1996) Br. J. Cancer 73, 1237-1240.
[18] Schroder, J., Schroder, E. and Cann, H.M. (1977) Hum. Genet. 38, 91.