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ARTICLE

Immunocytochemical Phenotyping of Disseminated Tumor Cells in Bone Marrow by uPA Receptor and CK18: Investigation of Sensitivity and Specificity of an Immunogold/Alkaline Phosphatase Double Staining Protocol

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SUMMARY Phenotyping of cytokeratin (CK)18-positive cells in bone marrow is gaining increasing importance for future prognostic screening of carcinoma patients. Urokinase-type plasminogen activator receptor (uPA-R) is one example of a potential aggressive marker for those cells. However, a valid and reliable double staining method is needed. Using monoclonal antibodies against uPA-R and CK18, we modified an immunogold/alkaline phosphatase double staining protocol. UPA-R/CK18-positive tumor cell controls exhibited black uPA-R staining in 15–80% of cases and red CK18 staining in almost 100% of tumor cells. Isotype- and cross-matched controls were completely negative. Bone marrow from healthy donors was always CK18-negative. Reproducibility of CK18-positive cell detection was estimated in a series of specimens from 61 gastric cancer patients comparatively stained with the single alkaline phosphatase–anti-alkaline phosphatase (APAAP) and our double staining method (10^6 bone marrow cells/patient). In four cases, double staining could not reproduce CK18-positive cells. In 34 cases it revealed fewer or equal numbers, and in 23 cases more CK18-positive cells than the APAAP method. Overall quantitative analysis of detected cell numbers (838 in APAAP, range 1–280 in 10^6; double staining 808, range 0–253) demonstrated relative reproducibility of APAAP results by double staining of 97%. Correlation of results between both methods was significant ($p<0.001$, linear regression). Sensitivity of double staining tested in logarithmic tumor cell dilutions was one CK18-positive cell in 300,000. Specific uPA-R staining was seen on CK18-positive cells in bone marrow from 29 of 61 patients, and also on single surrounding bone marrow cells. To test the specificity of this staining, bone marrow cytospins from 10 patients without tumor disease were stained for uPA-R with the APAAP method. uPA-R expression was confirmed in all 10 cases, with a mean of 6.5% uPA-R-positive cells in 1000 bone marrow cells (SEM 1.2%). These results suggest that our double staining protocol is a sensitive, reproducible, and specific method for routine uPA-R phenotyping of disseminated CK18-positive cells in bone marrow of carcinoma patients. (J Histochem Cytochem 45:203–212, 1997)

KEY WORDS
immunogold/APAAP double staining
methodological aspects
uPA receptor
CK18-positive cells
bone marrow
phenotyping

Minimal residual tumor disease in solid epithelial cancers has been indicated during the last years by investigations of disseminated tumor cells in bone marrow identified by the marker cytokeratin 18 (CK18), a cytoskeletal component of simple epithelial and carcinoma-derived cells (Moll et al. 1982). The sensitivity and specificity of this marker against the mesenchymal background of bone marrow cells have been repeatedly demonstrated (Pantel et al. 1994; Pantel et al. 1993b; Riesenberg et al. 1993; Lindemann et al. 1992; Schlimok et al. 1987). Correlation with clinical prog-

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nosis (Jauch et al. 1996; Pantel et al. 1993a; Lindemann et al. 1992; Moss et al. 1991; Berger et al. 1988) and demonstration of dynamic postoperative development after curative tumor resection of CK18-positive cells in bone marrow being associated with later clinical outcome (Heiss et al. 1995a) suggest the biological relevance of these cells with clinical implications.

Our earlier results regarding the correlation of urokinase-type plasminogen activator receptor (uPA-R) on those cells, with their postoperative quantitative increase (Heiss et al. 1995a), also point to the clinical importance of their phenotypic characterization. uPA-R as a central representative of the urokinase system, a pattern of factors known to be involved in tumor-associated proteolysis and potentially to represent a tumor cell's invasive capacity (Blasi 1993; Moller 1993), may indicate aggressive phenotypes of disseminated tumor cells. Other parameters also, such as the tyrosin kinase receptor Erb-B2 (Pantel et al. 1993b) which is potentially associated with aggressive tumor cell growth, proliferation antigens (Pantel et al. 1993b), or tissue-specific antigens such as prostate-specific antigen (PSA) (Riesenberg et al. 1993), have already been identified on CK18-positive cells in bone marrow and may help in estimating their biological properties and tumor cell identity.

For reliable phenotyping of disseminated tumor cells, an immunocytochemical double staining method that can detect CK18-positive cells against the mesenchymal background of bone marrow with high sensitivity and reproducibility is necessary. The second antigen should be unequivocally identified as to its cellular localization, in good contrast to CK18 staining and without any crossreactivity.

Riesenberg et al. (1993) introduced a combination of immunogold staining with the immunocytochemical alkaline phosphatase (AP) technique for detection of PSA on disseminated CK18-positive cells in bone marrow of prostate cancer patients. We modified this double staining method for identification of uPA-R on disseminated tumor cells and, as stated, the first clinical results with this new protocol have recently been presented (Heiss et al. 1995a). The aim of the present study was to demonstrate the methodological power of our method. Therefore, we applied our modified double staining protocol for identification of uPA-R and CK18 to a series of 61 gastric cancer patients who had ostensibly exhibited a positive CK18 bone marrow status at surgery, as analyzed with the internationally accepted APAAP (alkaline phosphatase–anti-alkaline phosphatase) method (Cordell et al. 1984). We also applied it to logarithmic tumor cell dilutions and to bone marrow of individuals without malignancy. In the following, we demonstrate our results concerning the methodological aspects of our double staining technique, introducing this method as a sensitive and reproducible application for routine phenotyping of disseminated tumor cells in carcinoma patients.

Materials and Methods

Patients

Bone marrow was taken from 219 patients who underwent surgery for gastric cancer. As a first step, APAAP staining was performed to screen those patients for CK18-positive cells in bone marrow. A total of 61 patients with positive CK18 results in APAAP and at least 10^6 bone marrow cells left for a second screening with our double staining method were involved in the present study.

Bone Marrow Aspirates

Bone marrow was taken intraoperatively from both iliac crests (5 ml each) and heparinized. Immediately after aspiration, the bone marrow underwent Ficoll–Hypaque density centrifugation (Loos and Roos 1974) (density 1.077; Biochrom, Berlin, Germany) for isolation of mononuclear cells (2000 g/25 min). The interphase fraction was washed twice in PBS, resuspended to a final concentration of 10^6 cells/ml, and cytocentrifuged on glass slides (10^5 cells/slide). After air-drying for 12–24 hr, the preparations were stained immediately or stored at −80C.

Immunocytochemical APAAP Staining

APAAP staining for CK18-positive cells (Cordell et al. 1984) was done as a prerequisite to a later investigation with the double staining procedure. A total of 10^6 cells/patient were analyzed. After fixation (acetone, 7 min) and incubation with 20% AB serum/PBS for 25 min to reduce unspecific staining, cells were incubated with MAb CK2 against cytokeratin component 18 (Boehringer, Mannheim, Germany; 4 μg/ml, IgG_1, 45 min), rabbit anti-mouse bridging antibody (Dako, Hamburg, Germany; 3 mg/ml, 1:25, 30 min), and monoclonal mouse APAAP complex (Dako; 0.17 mg/ml, 1:100, 30 min) in a moist chamber. Specifically bound AP was visualized with a solution containing 0.2 mg/ml naphthol AS-MX phosphate (dissolved in dimethyl-formamide; Sigma, Deissenhofen, Germany), 1% Fast Blue BB salt 1 mg/ml (Sigma), 0.1 M Tris buffer (pH 8.2), and 0.25 mg/ml levamisole (Sigma) to block endogeneous phosphatase activity. Each assay was controlled negatively by one slide stained with nonspecific IgG_1 (MOPC 21; Sigma) instead of CK2 and a slide of bone marrow from a healthy donor stained for CK18, and positively by a slide of the CK18-positive colon cancer cell line HT-29 resp. gastric cancer cell line KATO-III.

Immunocytochemical Double Staining

Biotinylation of MAb CK2 was done by dissolving 1 mg/ml CK2 in 25 μg d-biotinyl-ϵ-aminocaproyl-*N*-hydroxysuccinimide (Boehringer) and 50 μl dimethyl-formamide (DMF), overnight incubation, and membrane ultrafiltration with a Centricon centrifugal microconcentrator (Amicon; Witten, Germany) to separate DMF and uncoupled biotin (Bonnard et al. 1984; Blatt and Robinson, 1968).

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A modified double staining protocol based on the method described by Riesenberg et al. (1993) was applied in a moist chamber. Slides were fixed in acetone for 7 min and incubated in 20% AB serum/PBS for 25 min. All antibodies were diluted in 10% AB-serum/PBS, and each incubation step was followed by thoroughly washing the slides three times in PBS. A mouse MAb against uPA-R specifically recognizing membrane-bound and intracellular uPA-R (#3936, 10 μg/ml, $IgG_{2\alpha}$; American Diagnostica, Greenwich, CT) was incubated for 60 min, followed by gold-labeled goat anti-mouse antibody for 30 min (0.08 mg/ml, 1:50, Auroprobe One Reagent; Amersham, Braunschweig, Germany). To avoid crossreactions, 10% mouse serum/PBS (Dako) was applied for 25 min.

The second part of the double staining was performed using biotinylated CK2 MAb (10 μg/ml, 45 min) and AP-conjugated streptavidin (1.1 mg/ml, 1:100, 30 min; Jackson ImmunoResearch, West Grove, PA). In contrast to Riesenberg et al.(1993), this was not followed by postfixation in glutaraldehyde.

Visualization of specifically bound CK2 was done with new fuchsin dye (0.40 mg/ml; Serva, Heidelberg, Germany), sodium nitrite (0.04 mg/ml; Merck, Darmstadt, Germany), levamisole (0.36 mg/ml; Sigma), 0.2 M Tris buffer (pH 8.7), and naphthol AS-BI phosphate (0.08 mg/ml; Sigma) dissolved in DMF.

After washing the slides thoroughly in bidistilled water, specifically bound 1-nm colloidal gold particles (uPA-R staining) were visualized by silver enhancement under microscopic control. Equal volumes of inducer and enhancer of a silver enhancement kit (Amersham) were mixed and immediately incubated at room temperature for a maximum of 40 min. The silver kit was completely exchanged after 20-min incubation to avoid unspecific precipitation of silver granules. Slides were washed in bidistilled water and mounted with Kaiser's glycerol gelatin (Merck).

For comparison of sensitivity between this method and the original protocol of Riesenberg et al.(1993), preparations of the tumor cell dilutions (see below) were additionally stained using 8 μg biotinylated CK2 instead of 10 μg 5% mouse serum/PBS for 20 min and 2% glutaraldehyde/PBS (10 min) for postfixation after the incubation of streptavidin.

Slides of colon cancer cell lines SW403 and HT29 (ATCC; Rockville, MD) treated under the same conditions served as positive controls. For isotype crossreactivity controls, MAb CK2 was replaced by murine IgG_1 (MOPC21; Sigma) and MAb against uPA-R by murine $IgG_{2\alpha}$ (UPC 10; Sigma) in equimolar protein concentrations. Two other slides were stained without the first bridge and the second primary antibody, and vice versa. Two further slides underwent the staining procedure without the first second bridge. Bone marrow of a healthy donor served as another control.

Tumor Cell Dilutions for Determination of Sensitivity

The sensitivity of our method regarding detection of CK18-positive cells required testing in comparison to the original protocol (Riesenberg et al. 1993). Therefore, SW403 tumor cells were logarithmically diluted in peripheral blood leukocytes (PBL) to 1 tumor cell in 10^6, with additional dilutions of 1:200,000, 1:300,000, and 1:500,000. Solutions were cytocentrifuged (10^5 cells/slide) as described previously. Dilutions were analyzed for number of tumor cells detected and intensity of tumor cell staining. The results of this investigation were confirmed twice by two further replicates of the dilution.

Modification of the Double Staining Protocol

The original protocol of Riesenberg et al. (1993) was initially tested on 10 slides with 10^5 cells each of each tumor cell dilution described above. This revealed that the sensitivity for detecting CK18-positive cells was not optimal (1:10,000). Therefore, 11 further experiments were performed to improve the sensitivity, each confirmed by one further replicate of the tumor cell dilution: CK2 antibody was tested in two higher concentrations (10 and 12 μg/ml), streptavidin was increased to 1:80 and 1:50 in two other experiments, and glutaraldehyde postfixation was omitted in another series. Background staining was seen with the higher CK2 and streptavidin concentrations. Therefore, in four further tests mouse serum was increased to 10% compared to 5%, in combination with higher antibody and streptavidin concentrations. Background staining was not present at 10 μg/ml CK2 antibody, and the staining intensity of cells was improved. However, the sensitivity in dilutions was not significantly increased. Background was still seen with higher streptavidin and 12 mg/ml CK2. Therefore, we settled on a protocol with 10 μg/ml CK2 and streptavidin 1:100.

The sensitivity increased to 1:100,000 with the omission of glutaraldehyde postfixation. This was not diminished in a second experiment with 10% mouse serum and omission of postfixation.

A final protocol was therefore established with 10 μg/ml CK2, 10% mouse serum, and omission of glutaraldehyde. Testing at logarithmic tumor cell dilutions comparing the modified protocol with the original was now done according to the preceding paragraph.

Investigation of uPA-R Expression in Normal Bone Marrow Cytospins

Expression of uPA-R in cultured normal bone marrow stimulated with cytokines has been recently described (Plesner et al. 1994). To ensure the specificity of our double staining protocol, which detected uPA-R in normal control bone marrow (see Results), we investigated expression of uPA-R in bone marrow from 10 control patients (10^6 cells each) from our surgical department using the single APAAP protocol described above and MAb against uPA-R (American Diagnostica). The patients enrolled underwent surgery for nonmalignant diseases: one for abdominal aortic aneurysm, two for arterial occlusion, two for inguinal hernia, one for leiomyoma, and one for rectal adenoma. One patient underwent bone marrow biopsy because of hemolytic anemia, and one healthy member of our clinic gave informed consent for bone marrow donation. A total of 1000 bone marrow cells per patient were counted representatively for detection of uPA-R.

Analysis of Staining Results

All slides (including tumor cell dilutions) were coded and independently analyzed by two blinded investigators. Bone marrow preparations from patients were screened without knowledge of patient identity or stage of disease. Cell num-

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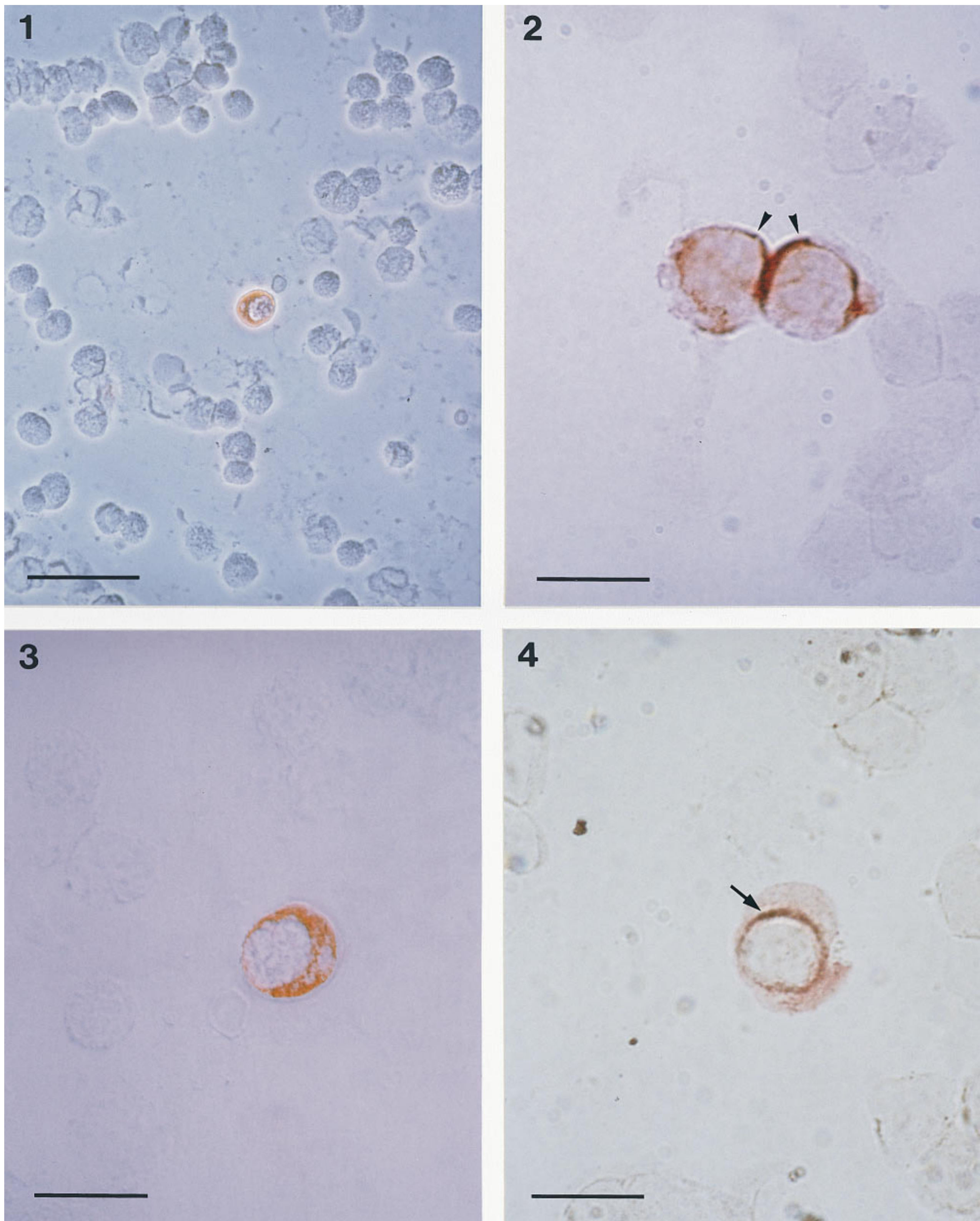

Figure 1 Example of a CK18-positive cell in bone marrow at 1:400 magnification, illustrating easy detectability of positive cells with our method even at low power. Bar = 50 μm.

Figure 2 Two cells expressing uPA-R (black, arrowheads) and CK18 (red). Original magnification × 1000. Bar = 20 μm.

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bers detected by double staining were counted independently from single APAAP results.

Statistical Analysis

A correlation diagram (scatter plot) with calculation of the regression line and linear regression analysis (level of significance $p<0.05$) was applied for estimation of correlation between CK18-positive cell numbers detected in APAAP–Fast Blue and the double staining method, using the EDA statistical software package (Department of Medical Information, Biometry and Epidemiology, Klinikum Grosshadern, Munich, Germany). This program was also used for calculation of means, standard deviations (SD), and standard errors of the mean (SEM).

Results

Staining Results and Controls

CK18-positive cells were easily detected by deep brownish-red staining of the cell cytoplasm, even at low magnifications (Figures 1 and 3). Bone marrow from healthy donors was routinely stained as a negative control in each assay, and never exhibited CK18-positive cells.

Expression of uPA-R on CK18-positive cells was indicated by black-grained linear staining of cell membranes (Figure 2). In some cases the staining appeared adjacent to nuclear membranes (Figure 4). Between 10 and 50 of 1000 surrounding bone marrow cells revealed specific staining for uPA-R. The specificity of this phenomenon was investigated in bone marrow from 10 donors with nonmalignant diagnoses (see below). UPA-R expression by CK18-positive cells was detected in 29 of the 61 patients investigated.

The tumor cell lines SW403 and HT29 showed almost 100% CK18-expression, with uPA-R expression between 15–80%, depending on the culture passage. A maximal percentage of uPA-R expression could be found at the beginning of tumor cell culture.

All isotype controls were negative, and neither specific nor unspecific red or black staining was detected.

UPA-R Expression in Normal Bone Marrow

To prove the specificity of uPA-R staining of bone marrow cells in our double staining method, we also stained bone marrow cytospin preparations from 10 patients with nonmalignant disease for uPA-R, using the established APAAP–Fast Blue protocol (Cordell et al. 1984). Patients with leukemia or lymphoma were excluded from this investigation because potential bone marrow infiltration with atypical leukocytes probably expressing uPA-R (Plesner et al. 1994; Wilson et al. 1983) could lead to false-positive results. uPA-R was detected in bone marrow of all the patients included in this study. Table 1 shows the clinical diagnosis, number of uPA-R-positive bone marrow cells in 1000, and the corresponding percentages. The mean percentage of uPA-R-expressing bone marrow cells was 6.5% (±1.2%).

Sensitivity of the Double Staining Protocol

The sensitivity of detection of expected CK18-positive cells was tested with tumor cell dilutions (SW403/PBL) described above (see Materials and Methods) using the original staining protocol of Riesenberg et al. (1993). Tumor cells in expected quantities and easily visible staining could be detected only up to a dilution of 1:10,000 and at very reduced staining intensity in the dilution of 1:100,000 (Table 2). Therefore, we modified this protocol step by step, testing each modification with the tumor cell dilutions described (see Materials and Methods).

With a double staining protocol using 10 μg/ml biotinylated MAb CK2 and omitting the postfixation step with glutaraldehyde, we could optimize sensitivity up to 1 tumor cell in 300,000, with good visibility of stained cells. The results of this modified protocol compared to the original are shown in Table 2, which gives means and standard deviations of cell counts found in 10 slides with 10^5 bone marrow cells each. The results were confirmed by two replicates of the tumor cell dilution, one revealing no positive cells at dilutions of 1:100,000 and higher with the original protocol and 4 cells in 10 slides at 1:300,000 with the modified method, the second showing three hardly visible positive cells in 10^6 at 1:100,000 with the original (higher dilutions negative) and two clearly detectable cells at 1:300,000 with the modified protocol, with expected tumor cell numbers in all lower dilutions.

Estimation of Reproducibility of CK18-positive Cell Detection

Of our 61 patients, 1 million bone marrow cells (10 slides with 10^5 cells each) were stained with our modified double staining protocol and with the APAAP–Fast Blue method as well, as positive CK18 counts in the single APAAP method were defined as prerequisite for additional double staining. Therefore, comparison between quantitative CK18-positive results of both methods was expected to allow an estimation of the reproducibility of our double staining technique.

Figure 3 CK-18-positive cell in bone marrow without uPA-R staining. Original magnification × 1000. Bar = 20 μm.

Figure 4 UPA-R detection (black) adjacent to the nuclear membrane (arrow) of a CK18-positive cell. Original magnification × 1000. Bar = 20 μm.

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Table 1 Expression of uPA-R in cytospins of normal bone marrow[a]

Diagnosis	Positive cells per 1000	Percentage
Abdominal aortic aneurysm	64	6.4
Arterial occlusion	25	2.5
Rectal adenoma	145	14.5
Inguinal hernia	36	3.6
Leiomyoma	49	4.9
Inguinal hernia	112	11.2
Varicosis	79	7.9
Arterial occlusion	10	1.0
Hemolytic anemia	65	6.5
Healthy donor	61	6.1
Mean ± SD	64.6 ± 38.3	6.5 ± 3.8

[a]Expression of uPA-R in cytospin preparations of normal bone marrow. A total of 10^6 cells were stained per patient and 1000 of them were counted representatively for uPA-R staining.

Detailed quantitative results of both methods are given in Figure 5 for each of the patients. It shows comparison of cells detected in APAAP (y-axis) with double staining (x-axis).

In four cases (7%), our double staining method was unable to detect CK18-positive cells again. Therefore, the reproducibility of qualitative cell detection in APAAP by double staining was estimated as 93%.

In 56% of cases (n=34), double staining found fewer or equal cell numbers. In the four patients with zero redetection of cells by double staining, 7 cells had been found altogether with the APAAP technique (for the single cases range 1–3 cells in 10^6, mean 2.0, SD 0.8). In six further patients, APAAP detected 17 cells altogether (range 1–4 in 10^6 in the individual case, mean 2.8, SD 1.2), and the double staining method could redetect the same cell numbers again. In 24 patients, APAAP staining revealed 653 cells in total (range 2–280 in 10^6 per individual, mean 27.2, SD 54.2), and with 495 cells altogether double staining redetected fewer CK18-positive cells (range 1–253 in 10^6, mean 21.5, SD 48.9). In 44% of cases (n=27) double staining found more CK18-positive cells in bone marrow than did the APAAP–Fast Blue method. APAAP had revealed a total of 161 cells in these 27 patients (for each case range 1–40 in 10^6, mean 6.0, SD 8.0), and the double staining method found 296 cells (range 1–53 in 10^6, mean 11.0, SD 10.9). Overall, a relative quantitative CK18-positive cell detection of 97% (808 cells in total) compared to the APAAP method (838 cells in all 61 patients) was seen.

CK18-positive cell counts of the two methods were positively correlated (Figure 5) with r=0.76 and $p<$ 0.001 (linear regression analysis).

Because variability of CK18-positive cell contents among cytospins potentially detectable by immunocytochemical methods should increase with a decrease in overall CK18-positive cell numbers in 10^6 and should be highest in cases with very low CK18-positive cell load, we additionally calculated the relative reproducibility of cell numbers for the 33 patients with 5 or more CK18-positive cells in single APAAP separately. Even in these cases with potentially more stable CK18-positive cell distribution, the double staining protocol revealed 94% (in total 735 compared to 783) of CK18-positive cells detected in single APAAP staining.

Discussion

Our double staining method combines an immunoenzymatic step (streptavidin–biotin–APAAP) with an immunogold-method of high sensitivity. Danscher (1981) and Holgate et al. (1983) first introduced immunogold techniques that could be used for light microscopic antigen detection by gold enhancement with silver precipitation. By further reducing the size of antibody-conjugated gold particles to 1 nm, increasing the penetration into cells (De Valck et al. 1991; Scopsi and Larsson 1985), the use of gold-conjugated bridging antibodies instead of direct marking of antigens (Scopsi and Larsson 1985), and modification of precipitating silver salts (Scopsi and Larsson 1985), the sensitivity

Table 2 Comparison of sensitivity between the original and the modified staining protocol[a]

		Cells detected with original protocol			Cells detected in modified protocol		
Cell dilution	Cells expected in 10^5	Mean	SD	%	Mean	SD	%
1:10	10,000	8700	1077	87.0	9100	1221	91.0
1:100	1000	891	95	89.1	931	100	93.1
1:1000	100	91.8	7.0	91.8	93.7	6.9	93.7
1:10,000	10	7.9	1.2	79.0	9.6	1.6	96.0
1:100,000	1	0.7[b]	0.6[b]	70.0[b]	0.9	0.7	90.0
1:200,000	0.5	0.1[b]	0[b]	20.0[b]	0.3	0.5	60.0
1:300,000	0.3	0	0	0	0.2	0.4	66.7
1:500,000	0.2	0	0	0	0	0	0
1:1,000,000	0.1	0	0	0	0	0	0

[a]Comparison of CK18-positive cell sensitivity between the staining protocol of Riesenberg et al. (1993) and our modification without glutaraldehyde postfixation (see Materials and Methods). For this study, SW403 tumor cells were logarithmically diluted in PBL, and 10 slides with 10^5 cells each stained with both protocols. Means and standard deviations are given for the results of 10 slides stationed with each method.
[b]Results of the original protocol (dilutions 1:100,000 and 1:200,000) indicate staining at very reduced intensity levels.

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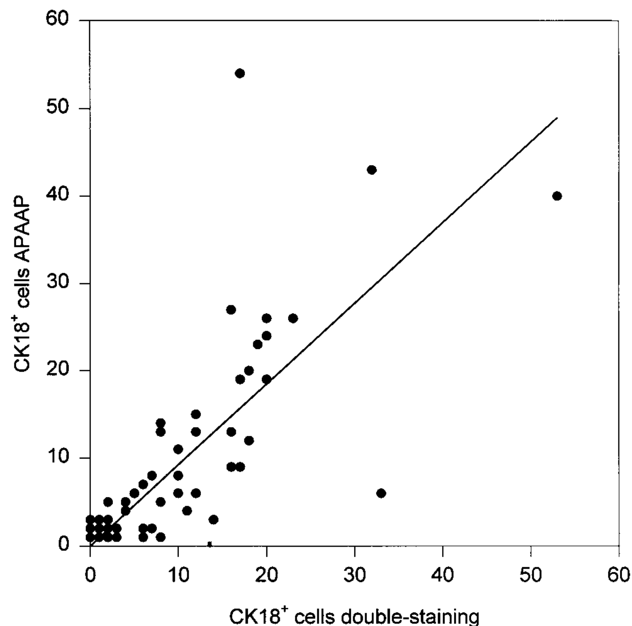

Figure 5 Correlation diagram (scatter plot) showing the association between CK18-positive cell counts in single APAAP and in our double staining method. Each point in the graph represents one patient, showing the number of positive cells detected with double staining (x-axis) and with single APAAP (y-axis) for the individual case. One patient with 280 cells in APAAP and 253 cells in double staining is not shown in the graph. Correlation coefficient (r=0.76) and inclination of the regression line (y=0.88X+0.08) show positive association of APAAP and double staining results. Correlation is significant, with p<0.001 (linear regression analysis).

of this method was optimized. This led to combination of the immunogold method with immunoenzymatic procedures for simultaneous marking of two antigens (Sako et al. 1986; Scopsi and Larsson 1985; Van den Pol 1984; Gu et al. 1981).

The advantage of this combination compared to double enzymatic methods is avoidance of color mixing, which often leads to false-negative results and inability to clearly identify subcellular localizations of the two antigens investigated (Riesenberg et al. 1993; Mason and Woolston 1982). Especially in the case of the CK18 antigen, low amounts of the second antigen, which often occur in marking of membrane-bound receptors such as uPA-R, are often masked by strong CK18 staining. This, however, is prevented by application of immunogold for investigation of the second antigen, which is reliably detected even at low amounts, with good contrast to red CK18 staining and clear identification of localization within the cell (Riesenberg et al. 1993).

Compared to immunofluorescence, our method enables time-permanent preparations with visibility of colors for years, and is more cost-effective (Antica et al. 1986; Mason and Woolston 1982; Rathlev et al. 1981). Compared with immunoautoradiographic methods (Antica et al. 1986), it avoids exposure to harmful radiation, and visualization of specific antigen marking takes about 1 hr compared to several days in immunoautoradiography (Antica et al. 1986).

An alternative to immunocytochemical double labeling could be provided by flow cytometry, which also enables identification of more than one antigen even quantitatively by multiplex labeling at sensitivities reaching 1 cell in 1,000,000 (Shapiro 1995). However, for multiplex labeling in flow cytometry, the cell types involved should be well characterized for unequivocal identification and differentiation, and intermediate cell forms should not be present (Shapiro 1995). These intermediate cell forms, however, are a major characteristic of bone marrow, a fact that could limit applicability of this method to identification of tumor cells in marrow samples. Furthermore, the use of fixatives, often necessary to preserve cells for a few days until screening is done, can lead to artifacts such as autofluorescence (Shapiro 1995). Moreover, in contrast to flow cytometry, double immunocytochemistry provides preparations that can be reinvestigated years after staining.

The sensitivity of our double staining protocol was tested with logarithmic dilutions of tumor cells in comparison to Riesenberg's original protocol. It was seen that sensitivity (also considering easily visible staining intensity) could be improved from 1:10,000 to 1:300,000 by omitting postfixation with glutaraldehyde, because higher CK2 antibody concentration (10 μg/ml compared to 8 μg/ml in the original protocol) alone had resulted in only poor improvement of sensitivity (data not shown). The fixation mechanism of glutaraldehyde is known to be alcylation of sulfhydryl and COOH groups of cell membrane proteins, which potentially decreases the permeability of cell membranes to different molecules (Boenisch 1989). It is conceivable that it also inhibits substrates of AP specifically bound to intracellular CK18 from permeating, thus lowering the intensity of the red staining reaction. In addition, Guesdon et al. (1979) described decreasing activity of avidin, which is part of the second step in our double staining method, by application of glutaraldehyde. This can explain the improvement of sensitivity by omitting the glutaraldehyde step of Riesenberg (Riesenberg et al. 1993). Background staining as described by Guesdon et al. (1979) was not enhanced in our modification, and subcellular antigen localization could easily be identified despite the omission of postfixation.

Comparison of CK18-positive cell numbers detected with our double staining method with APAAP–Fast Blue results should allow estimation of reproducibility. In only four cases (CK18-positive cell numbers between 1 and 4; see Table 2) did our double staining protocol fail to reproduce positive cells. Summarizing

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cell numbers of the 61 patients investigated, our double staining protocol was able to detect 97% of CK18-positive cells in APAAP. In 44% of cases, the double staining method demonstrated even more cells than the APAAP method. This can certainly be explained by the variability of CK18-positive cell distribution within the cytospin preparations, especially in patients with low CK18-positive cell counts, as summarized from investigation of 10 cytospins with 10^5 bone marrow cells each. Leaving out those cases with very low CK18-positive cell counts in single APAAP (<5 in 10^6), we tried to decrease this influence, because with higher CK18-positive cell numbers the variability of detection due to incidental cell distribution between cytospins should become more stable. Even here, the double staining method detected 94% of the cell number in single APAAP, indicating good reproducibility. Nevertheless, it should be emphasized that this comparison is an approximation for the true reproducibility of our method, because different cytospins with potentially different CK18-positive cell numbers had to be stained with single APAAP and the double staining protocol.

Because the immunogold step of our method is known to be highly sensitive (De Valck et al. 1991; Scopsi and Larsson 1985; Holgate et al. 1983), further improvement of uPA-R marking by immunogold was not the aim of our study. Nevertheless, further investigations with different uPA-R antibodies compared to MAb 3936 used here could lead to optimization of uPA-R sensitivity, because specific binding of this antibody may be lowered by high molecular weight uPA or pro-uPA complexes (Chucholowski et al. 1992). Preincubation with iodine or other oxidating substances (Holgate et al. 1983), thus enhancing the interactiveness of immunogold particles, may also lead to optimization of the immunogold step.

uPA-R marking was present not only on CK18-positive cells but also on bone marrow cells. False-positivity of this phenomenon was excluded by staining of normal bone marrow for uPA-R with the established APAAP method. Here, expression of uPA-R could be demonstrated for a mean of 6.5% of cytospin bone marrow cells. From the literature it is established that uPA-R is expressed on blood leukocytes (e.g., monocytes, neutrophilic granulocytes, activated T-cells (Kramer et al. 1994; Blasi 1993), and bone marrow cytospins necessarily contain a certain amount of sinusoidal blood. This in itself provides an explanation for our findings. In addition, Plesner et al. (1994) described that cytokine stimulation of CD34-positive bone marrow stem cells, which initially were uPA-R-negative, led to 40% uPA-R-expressing bone marrow cells with advancing cell differentiation in vitro. Discrepancy between Plesner's and our mean percentages of uPA-R-positive bone marrow cells may, in our opinion, be explained by differences between the physiological in vivo situation and experimental in vitro stimulation.

Other authors, using the same double staining method, also confirm the specific marking of the immunogold step. Riesenberg et al. (1993) detected PSA exclusively on disseminated prostate tumor cells and not in bone marrow cells, which are known to be PSA-negative. The proliferation antigen Ki67 is known to be expressed in bone marrow (Gerdes et al. 1984), and Pantel et al. (1993b) described positive Ki67 staining of CK18-positive as well as surrounding CK18-negative bone marrow cells. In contrast, the same study did not detect immunogold staining of bone marrow cells for p120, a proliferation marker that is absent in bone marrow (Pantel et al. 1993b).

The specificity of our method is further supported by permanently negative isotype and cross-matched controls. Background staining could be reduced to minimum by extensive washing between the incubation steps, the importance of which is also confirmed by Holgate et al. (1983) and by Scopsi and Larsson (1985), who even postulate an increase of sensitivity through prolonged washing when polyclonal antibodies are used. Second, renewing the silver enhancement solution after a maximal time of 20 min also led to reduced silver background.

In our present investigation, uPA-R was mainly detected on cell membranes. In single cases of disseminated CK18-positive cells in bone marrow and also in tumor cell line-positive controls, staining for uPA-R was also localized on nuclear membranes. It is necessary in further investigations to verify this nuclear staining by electron microscopy. However, this finding is corroborated by the report of Bastholm et al. (1994), who describe paranuclear fluorescence patterns investigating uPA-R in the breast cancer cell line MDA-MB-231 by fluorescence microscopy. Jankun et al. (1993), using the same MAb 3936 against uPA-R as in our study, detected holonuclear staining in single breast carcinomas investigated immunohistochemically with the immunoperoxidase method. In this study, benign breast tumors stained in comparison in 33% of cases were uPA-R-negative, generally exhibited weaker staining intensities than the carcinomas, and never showed uPA-R localization in the nucleus. Therefore uPA-R expression in the nucleus might be a characteristic of malignancy, and further studies are desirable to verify this hypothesis.

In summary, our present investigation suggests a double staining protocol that allows highly sensitive characterization of disseminated CK18-positive cells in bone marrow, as well as good reproducibility, by use of an established method. The first results regarding correlation of uPA-R detection on those cells with later increasing CK18-positive cell counts and a worse prognosis for cancer patients (Heiss et al. 1995a,b) may

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lead to identification of aggressive phenotypes of systemically spread tumor cells with this marker. Therefore, the method may be helpful in evaluating the biological relevance of single individual cells to later occurrence of clinical metastasis, which could be particularly important for patients with macroscopically early tumor stages but evidence for subclinically spread tumor cells. Our method might have broad applications in patients with various forms of epithelial cancer and in differentiating disseminated tumor cells by patterns of other antigens.

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