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Overexpression of Nucleoside Diphosphate Kinase (Nm23) in Solid Tumours

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The product of the *nm23-H1* gene, reported to be related to the metastatic potential of tumour cells, was recently identified as the nucleoside diphosphate (NDP) kinase A (Gilles *et al.*, 1991, *J Biol Chem*, 266, 8784–8789). An analysis of the enzyme by activity measurement and immunological techniques using polyclonal antibodies raised against the NDP kinase A purified from human erythrocytes, was performed on 39 human tissue specimens. Markedly increased activity and higher level of the protein were observed in extracts of solid tumours as compared to the corresponding normal tissues ($P < 0.01$). An intense immunolabelling of tumoral cells was observed in sections of the malignant tumours and of some but not all benign neoplasia. The staining is observed in non-invasive and invasive ductal breast carcinomas with or without lymph node involvement as well as in colon and cervix carcinomas and in a case of metastatic melanoma. Therefore, NDP kinase A level is increased in neoplastic tissues but no correlation with metastatic potential could be demonstrated.
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INTRODUCTION

THE *nm23-H1* GENE was originally identified by differential screening of a cDNA library with RNA from low and high metastatic clones of a murine melanoma cell line [1]. In studies on established cell lines and human breast tumours, reduced expression of the *nm23-H1* gene was correlated with a high metastatic potential [1–4]. A second human gene, *nm23-H2*, was recently identified and shown to encode a protein with 88% identity to the product of *nm23-H1* [5]. Nm23 was first proposed to be a nucleoside diphosphate kinase (NDP kinase) based on sequence homology with the *Dictyostelium discoideum* enzyme [6, 7]. It is also highly homologous to the product of the *Drosophila awd* gene in which mutations induce widespread developmental abnormalities [8]. The *awd* gene has also been shown to encode a nucleoside diphosphate kinase [9].

NDP kinases are ubiquitous enzymes playing a major role in the synthesis of nucleoside triphosphates other than ATP [10, 11]. It was recently shown that the enzyme from human erythrocytes is an hexamer made of two types of subunits (A and B) exhibiting distinct pI and electrophoretic behaviour [12]. Several active isozymes including the A6 and B6 homopolymers can be formed by the association of both polypeptides [13] and can explain the presence of several isozymes in the cell [10]. The identification of Nm23 as an NDP kinase was recently definitively confirmed by demonstration of the identity of the primary structure of the NDP kinase A and NDP kinase B of human erythrocytes [13] with the sequences deduced from *nm23-H1* and *nm23-H2* genes, respectively [4, 5].

The present study was undertaken to determine whether alterations in the expression of Nm23/NDP kinase occur in tumour cells as compared to normal tissue. Using biochemical and immunohistological approaches, we have measured the activity of NDP kinase in tissues extracts, and evaluated the level of the protein by western blot and immunohistochemistry. Specimen analysed included normal tissues, benign breast neoplasia, colon, breast and cervical carcinoma and a metastatic melanoma.

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MATERIALS AND METHODS

Materials

Nitrocellulose filters (BA-85 from Schleicher & Schuell) were used for western transfer. ATP, dTDP, NADH, phosphoenolpyruvate, phenylmethylsulphonyl fluoride (PMSF), nitro blue tetrazolium, 5-bromo-4 chloro-3-indolyl phosphate, antipain, benzamidine, diaminobenzidine and haematoxylin were obtained from Sigma. Pyruvate, sodium salt, was purchased from Fluka. Pyruvate kinase and lactate dehydrogenase were from Boehringer Mannheim. Swine serum, swine anti-rabbit antibodies and the PAP (peroxidase–antiperoxidase) solutions were obtained from Dakopatts (Denmark). The polyclonal anti-NDP kinase antibodies were raised in rabbits immunised with the purified A enzyme from human erythrocytes [13].

Tissues

Normal and neoplastic tissues were obtained from surgical samples of patients treated at the Institut Curie (Paris) but not undergoing chemotherapy or radiotherapy. Each sample was divided into two portions: one was processed for conventional histopathological diagnosis and immunohistochemical studies while the other was immediately frozen and kept at −70°C for enzymatic assays.

Preparation of the tumour extracts

All steps were performed at 4°C. The tumour pieces weighting from 0.2 to 0.5 g, were thawed in 5 to 10 volumes (w/v) of homogenising buffer (10 mmol/l Tris-HCl buffer, pH 7.5, containing 20% glycerol, 1 mmol/l EDTA, 1 mmol/l PMSF,

1 μg/ml antipain and 15 μg/ml benzamidine), minced with scissors and homogenised with a Polytron homogeniser (Luzern, Switzerland). The supernatant fractions were obtained by centrifugation of the homogenates for 15 min at 20 000 *g*. The protein concentration of the supernatants was estimated by the method of Bradford [14] using γ-globulin as standard.

NDP kinase activity measurement

NDP kinase activity was measured using a coupled pyruvate kinase-lactate dehydrogenase assay as described by Agarwal *et al.* [10] with minor modifications [6]. The assays were performed at 30°C in a 0.5 ml reaction mixture containing 50 mmol/l Tris HCl, pH 7.4, 50 mmol/l KCl, 6 mmol/l $MgCl_2$, 1 mmol/l phosphoenolpyruvate, 0.1 mmol/l NADH, 0.5 mmol/l ATP, 0.1 mmol/l dTDP, 2 units of pyruvate kinase and 2.5 units of lactate dehydrogenase. The reaction was started by the addition of 5 μl of each supernatant fraction either undiluted or after a 10-fold dilution in the homogenising buffer. The activity is expressed as units (U) per mg protein (1 unit is the amount of enzyme which catalyses the formation of 1 μmol of ADP per min). The reaction rate was linearly related to time and protein concentration. We checked that under our conditions other enzymic activities such as adenylate kinase or ATPase which could have biased our assay were negligible. This was expected due to the very high NDP kinase-specific activity [13] and the 100-fold to 1000-fold dilution of the samples in the assay medium.

Statistical analysis of the data

The activities of the different sets of tissues (normal, benign and malignant neoplasia) were compared using one way analysis of variance followed by the Tukey's multiple comparison test. The highest value of the malignant tumours set was discarded to fit the Bartlett test of homogeneity of variance.

Western blot identification of NDP kinase in tumour extracts

The supernatants of the tumour extracts and the purified NDP kinase were electrophoresed on a 12.5% SDS–polyacrylamide gel according to Laemmli [15]. The proteins were transferred to a nitrocellulose filter which was probed with polyclonal antibodies (5000-fold dilution), raised against the NDP kinase A purified from human erythrocytes [12, 13], and revealed with [^{125}I] iodinated protein A or by ECL western blotting detection (Amersham). Preadsorbtion of the anti-A antiserum for control experiments was achieved by preincubating 1 μl of the serum with 25 μg of the purified NDP kinase A for 24 h at 4°C.

Immunohistochemical studies

Histological sections were prepared from tissues fixed in 4% formaldehyde for 48 h then dehydrated and embedded in paraffin. For immunohistochemical techniques, 5 μm sections were dewaxed, rehydrated and treated for 20 min with normal swine serum to prevent non-specific binding. After removing the excess serum, sections were incubated for 30 min at room temperature with the polyclonal anti-NDP kinase A antibodies diluted 100-fold. After washing, sections were incubated with the swine anti-rabbit antibodies and, after an other washing step, incubated with the PAP solution. The peroxidase activity was detected by diaminobenzidine in the presence of H_2O_2. Sections were counterstained with haematoxylin for 30 s dehydrated and mounted in Eukitt.

Table 1. Expression of NDP kinase in normal and neoplastic tissues

Case	Tissue	Histology	NDP kinase (U/mg protein)	Immunological labelling	Metastatic nodes
1	Muscle	Normal tissue	0.12	ND	
2	Muscle	Normal tissue	0.22	−	
3	Thyroid	Normal tissue	0.10	ND	
2	Skin	Normal tissue	0.15	−	
2	Breast	Normal tissue	0.12	−	
4	Breast	Normal tissue	0.15	−	
5	Breast	Normal tissue	0.11	−	
6	Breast	Normal tissue	0.02	−	
4	LN	Normal tissue	0.27	−	
7	LN	Normal tissue	0.28	−	
8	Breast	Benign hyperplasia	0.17	+	
9	Breast	Benign hyperplasia	0.12	−	
10	Breast	Benign hyperplasia	0.51	++	
11	Breast	Benign hyperplasia	0.37	++	
12	Breast	Benign hyperplasia	0.09	ND	
13	Breast	Fibroadenoma	0.57	+++	
14	Breast	Fibroadenoma	0.17	+	
15	Breast	Fibroadenoma	0.49	++	
16	Breast	Fibroadenoma	0.07	+	
17	Breast	Fibroadenoma	0.06	−	
18	Breast	Intra ductal c.	1.93	++++	−
4	Breast	Invasive ductal c.	0.79	+++	+
7	Breast	Invasive ductal c.	0.54	+++	+
5	Breast	Invasive ductal c.	0.70	+++	−
6	Breast	Invasive ductal c.	0.32	++++	+
19	Breast	Invasive ductal c.	ND	++++	+
20	Breast	Invasive ductal c.	0.70	+++	+
21	Breast	Invasive ductal c.	0.45	++	−
22	Breast	Invasive ductal c.	0.57	++++	+
23	Breast	Invasive ductal c.	0.34	+++	+
24	Breast	Invasive ductal c.	0.57	+++	+
25	Breast	Invasive lobular c.	0.22	++	+
2	Breast	Invasive lobular c.	0.91	+++	+
26	Breast	Medullary c.	0.67	++	−
2	LN	Metastatic c.	0.50	++	+
27	Colon	Glandular c.	0.41	++++	+
28	Colon	Glandular c.	0.25	++	−
29	Cervix	Squamous cell c.	0.58	+++	+
30	LN	Metastatic melanoma	0.80	++++	+

Activity values are the mean of triplicate determinations and agreed within 5%. The histological labelling intensity was graded from undetectable (−) (Fig. 2E) to strong (++++) (Fig. 2G). LN = lymph node, c = carcinoma, ND = not determined.

RESULTS

NDP kinase activity was measured in the extracts of specimens of normal tissues and of benign and malignant tumours (Table 1). Immunohistochemical analysis was performed on a part of the same specimens using antibodies raised against the human erythrocyte NDP kinase A [13]. The specificity of the antibodies was confirmed by ELISA test using purified NDP kinase A (not shown) and by western blot (Fig. 1A). The results of biochemical and immunohistochemical analyses of 39 tissue specimens are reported in Table 1. A low NDP kinase activity [average value (S.E.) 0.15 (0.03) U/mg protein] was found in normal tissues, the highest values being observed in muscle and lymph nodes. In normal tissue samples, NDP kinase was not or

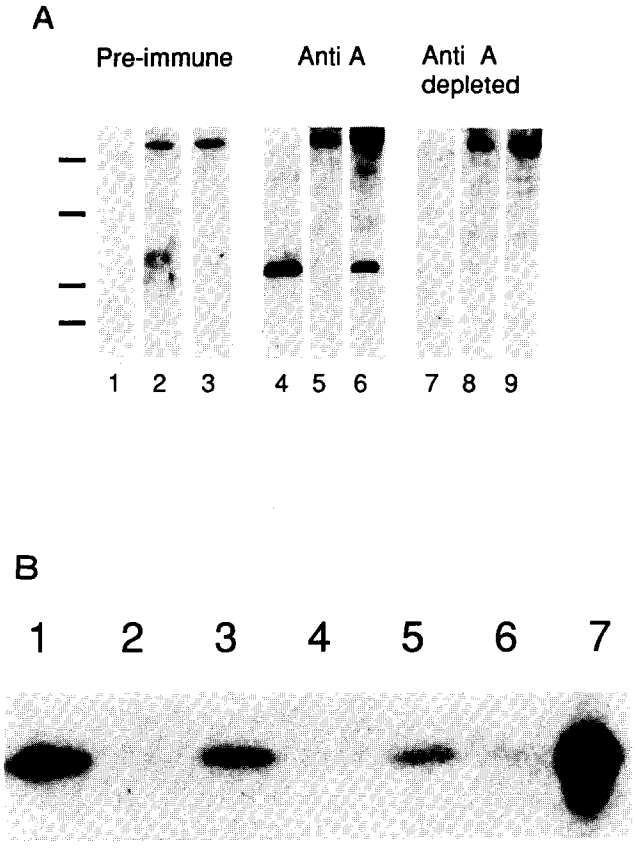

Fig. 1. Immunoblot analysis of different tissues. (A) Purified NDP kinase A (0.4 μg; lanes 1,4,7), normal breast (50 μg; case 2, lanes 2, 5,8) and invasive ductal carcinoma (50 μg; case not shown in Table 1; lanes 3,6,9) were probed with the preimmune serum, with the antiserum raised against the NDP kinase A and with the same antiserum preincubated with the purified enzyme. Molecular mass markers are from bottom to top: lysozyme (14 300); β-lactoglobulin (18 400); carbonic anhydrase (29 000) and ovalbumin (43 000). Case numbers refer to Table 1.

(B) Tissues (50 μg protein/lane) analysed were: lane 1, breast lobular carcinoma (case 2); 2, normal breast (case 2); 3, invasive ductal carcinoma (case 4); 4, normal breast (case not shown); 5, breast benign hyperplasia (case 12); 6, breast fibroadenoma (case 17). Lane 7 shows purified A/B NDP kinase (0.5 μg/lane) from human erythrocytes (apparent molecular weights: 20 500 for A and 19 000 for B, 13).

barely detectable by immunohistochemical techniques. A low level of NDP kinase was observed in benign breast tumours except in some proliferative hyperplasia. A markedly increased activity [0.55 (0.05)U/mg protein], was observed in malignant tumours, highly significantly different ($P < 0.01$) from the mean value of normal samples. Most values for malignant tumours were in the range 0.5 to 0.9 U/mg of protein, the highest activity (1.93 U/mg protein) being found in an intraductal breast carcinoma and the lowest value (0.22 U/mg protein) in a lobular adenocarcinoma. The NDP kinase is mainly cytosolic since the activities measured either in whole tissue extracts or in 105 000 *g* supernatants were similar (when calculated in units per ml of tissue extract) to those reported in Table 1 which were measured in 20 000 *g* supernatants (data not shown).

It should be stressed that an enhanced activity was observed in the primary tumour as well as in a metastatic axillary lymph node (Table 1, case 2). The ratio between the enzyme activities in the tumour and in the corresponding normal tissue extracts from the same patient could reach 8-fold. Variations in activities within tumour samples may result from difference in tumour cell number or could reflect differences between tumour stage or types. The immunostaining intensity in the neoplastic cells (Table 1, column 2) correlated with the NDP kinase activity, indicating that the increase in activity was due to an increased amount of the enzyme rather than to an activation of pre-existing enzyme.

Figure 1 shows an analysis by western blot of various tissues extracts using rabbit polyclonal antibodies raised against NDP kinase A. As shown in Fig. 1A, a strong band of the same size as purified NDP kinase A (apparent molecular mass: 20.5 kD) was detected in an extract of a breast carcinoma (lanes 4 and 6). Although the antiserum raised against the A polypeptide is also able to recognise to a lesser extent the B polypeptide [13], no band corresponding to the B subunit (apparent molecular mass: 19 kD) was detected (see also Fig. 1B). The extract from normal breast tissue showed no detectable band corresponding to NDP kinase (lane 5). Preimmune serum showed no reaction with a 20.5 kD band (lanes 1–3). The specificity of the antibodies was further demonstrated by showing that serum which had been pre-adsorbed with purified NDP kinase A could not react with the 20.5 kD species (lanes 7–9). A non-specific 45 kD band was detected in all tissue extracts also with preimmune serum (lanes 2,3,5,6,8,9).

A representative sample of several tissues extracts is analysed in Fig. 1B. Prominent strong 20.5 kD bands were observed with malignant tumour extracts (lanes 1 and 3). In contrast, extracts from benign hyperplasia exhibited a much weaker band (lanes 5 and 6) whereas normal tissue gave no or barely detectable signals under these conditions (lanes 2 and 4). In all cases examined, the increase in enzymatic activity was paralleled by an increase in the level of the polypeptide detected in the same cytoplasmic extract by western blot. Occasionally, a minor band corresponding to NDP kinase B was also recognised (data not shown).

Micrographs of representative sections are shown in Fig. 2. A strong cytoplasmic staining was observed in most malignant cells. When antiserum preadsorbed with purified NDP kinase A was used, no labelling was observed, demonstrating that the reaction of the antibodies on the immunohistochemical sections was specific for NDP kinase A (not shown). In breast tumours, an intense labelling was found in non-invasive as well as in invasive and metastatic ductal carcinomas (Fig. 2A, D). Similar labelling was also detected in metastatic cells of an invaded axillary lymph node (Fig. 2C). A strong staining of neoplastic cells was also observed in other types of malignancies, including a metastatic adenocarcinoma of the colon (Fig. 2F, G) and a metastatic melanoma (Fig. 2H). In fibroadenomas as in hyperplastic ductal structures, the immunoreactivity was weak or undetectable (Fig. 2E). However, some benign hyperplasia also exhibited labelling of epithelial cells (see Table 1).

DISCUSSION

The recent demonstration of the identity of the product of *nm23-H1* and NDP kinase A [13], validates the use of the NDP kinase A measurement to evaluate *nm23-H1* expression. Our data demonstrate that NDP kinase A/Nm23-H1 is highly expressed in tumours of various origin as compared to normal tissues.

NDP kinase activity had been detected in tumour cell lines [16, 17] but changes in activity have never previously been correlated to tumoral state and/or metastatic potential with the

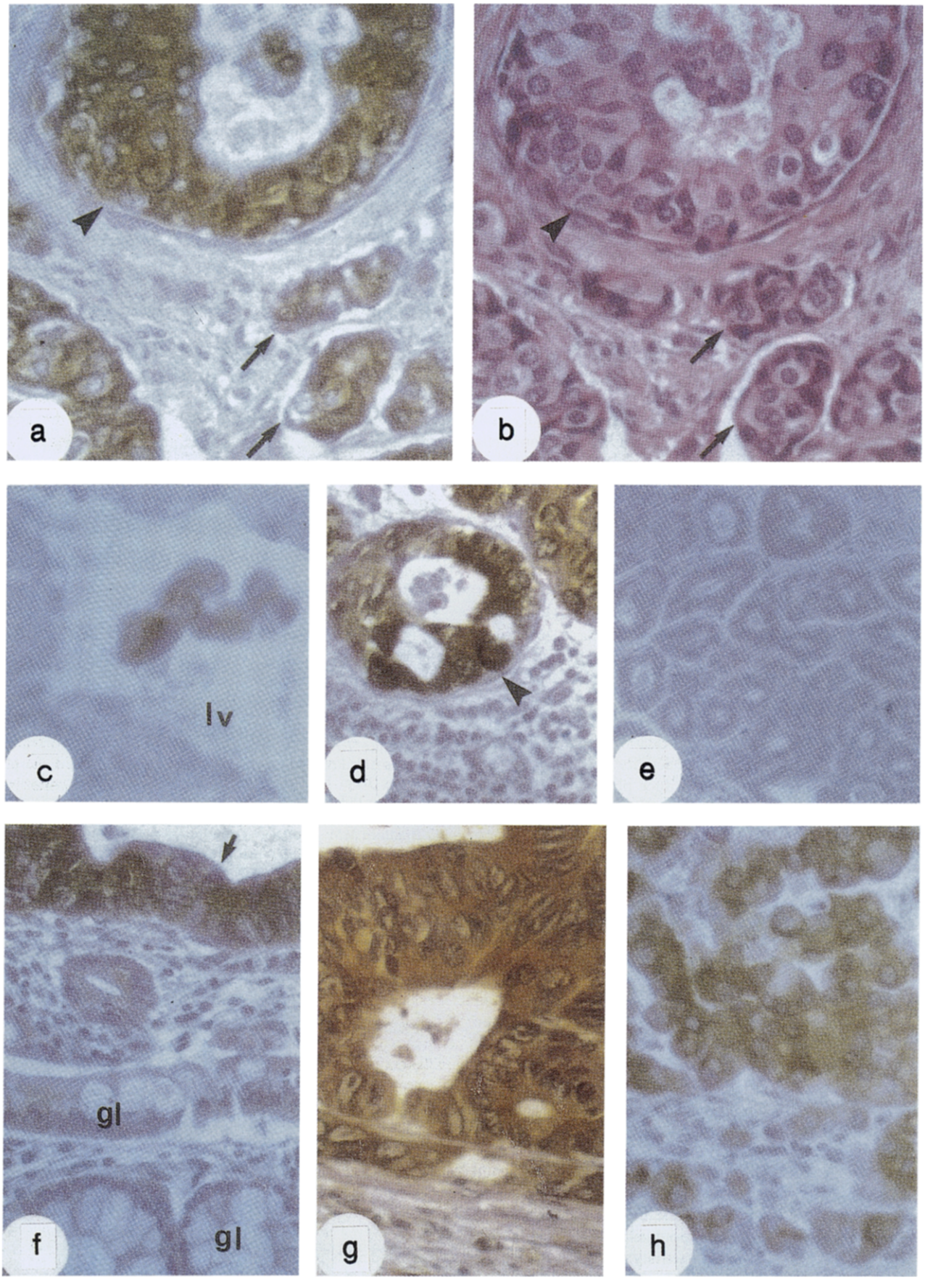

Fig. 2. Immunohistochemical staining with anti-human NDP kinase serum. (A) Infiltrating ductal carcinoma (case 19): strong labelling of the cytoplasm of malignant cells in infiltrative (small arrows) and intraductal components (large arrows). (B) Same field of a next section with standard staining. (C) Same patient, labelled metastatic cells in a lymphatic vessel (lv) of the axillary region. (D) Non-invasive ductal carcinoma with periductal lymphocytes (case 18): pronounced labelling of the neoplastic intraductal component and no labelling of lymphocytes. (E) Benign mastosis with hyperplasia (case 8): no obvious staining of the epithelial hyperplastic cells. (F) Invasive colon adenocarcinoma (case 27): strong staining of the neoplastic malignant cells (arrow) as compared to normal glandular epithelial cells (gl). (G) Same tumour. (H) Malignant melanoma invading a lymph node (case 30): strong labelling of most of the metastatic malignant cells. Magnifications: A, B, C, G and H × 650, F × 400, E × 160.

exception of the report of an increased level of autophosphorylated NDP kinase in tumoral versus normal human colon homogenates [18]. The mechanism of this increase and its effect on the neoplastic cellular metabolism is unknown. Since the sequence of the NDP kinase A [13] purified from human erythrocytes is identical to the primary structure deduced from the cDNA encoding the Nm23 protein found in tumours [4], the *nm23-H1* gene is unlikely to be mutated.

NDP kinases are crucial for the supplement of nucleoside triphosphates required for nucleic acid synthesis. Thus, an increased expression of this enzyme might be linked to proliferation, as has been shown for other enzymes of this pathway [19]. Indeed, the overexpression that we observed in some benign neoplasia might indicate a correlation with the proliferative state. It is worth noting that a correlation between elevated level of Nm23/NDP kinase and proliferation has been demonstrated in normal lymphoid cells induced to proliferate (D.R. Keim *et al.*). However, the fact that, in our study, most tumour cells seen on a section are labelled indicates that the increased level of NDP kinase is not restricted to a specific phase of the cell cycle. We have observed a preferential increase in the A subunit of the NDP kinase in tumour cells. This might result in the formation of NDP kinases composed of an altered ratio of A versus B subunits, which might alter yet unknown physiological processes.

A possible target of NDP kinase action could be the GTP binding proteins. In many systems, NDP kinases have been found associated with GTP binding proteins such as elongation factor [20], microtubules [9, 21], p21 [16] or $G_s\alpha$ [22], suggesting that they could be involved in processes like protein synthesis, tubulin polymerisation or signal transduction by channeling GTP [22–25] or by *in situ* phosphorylation of protein-bound GDP [26]. Thus, a high NDP kinase activity could possibly turn on GTP-regulated processes and induce pleiotropic effects on cellular functions. Further works are still needed to precise the role of NDP kinase in these processes.

We did not observe any reduction of the NDP kinase /Nm23 level correlated with metastatic potential in the set of tumours that we have analysed. The recent report of an increase of *nm23* mRNA in colon neoplasms as compared to normal mucosa, unrelated to metastatic potential [27] as well as a high protein level in neuroblastoma associated with advance stage disease [28] indicates that the correlation between *nm23* expression and metastatic potential might be restricted to certain cell lines or tumour types [29]. The reason for the discrepancy between previously reported decrease in *nm23* expression in breast tumours of high metastatic potential [3, 30] and our results are not clear at present. However, it should be noted that we have measured the presence of the protein, either by its activity or with polyclonal antibodies against the purified protein, whereas most of the preceding studies have relayed on RNA detection. It is known that the NDP kinase is a highly stable protein and therefore, a decrease in mRNA might not be reflected at the protein level as observed during the developmental cycle of *Dictyostelium discoideum* (V. W., unpublished).

In conclusion, the level and activity of NDP kinase are specifically increased in neoplastic cells of solid tumour as compared to normal tissues. Although the increase is variable, there is no clear correlation of a reduced increase in malignant tumours with metastatic spread. A large scale clinical study is currently in progress to try to correlate the prevalence of Nm23/NDP kinase with clinical and biological parameters of malignancy.

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Prolonged G2 Phase of Breast Cancer Cells and Chromosome Damage

B. Dutrillaux, M. Gerbault-Seureau, B. Zafrani and M. Prieur

5-bromodeoxyuridine incorporation was studied in metaphase chromosomes from 24 breast tumour specimens, including 23 adenocarcinomas. In these 23 cases, a slow cell cycle was observed, with a long (8 h) G2 phase. This slowing of the cell cycle, which was poorly related to the degree of polyploidy, was significantly related to the number of chromosome anomalies: the cell cycle was particularly slow when many rearranged chromosome were observed. These *in vitro* findings during the first cell cycle cannot easily be transposed to the *in vivo* situation. By analogy with Fanconi anaemia, in which both chromosome lesions and a long G2 phase are detected, a DNA repair defect and/or high DNA mutagenesis might exist in breast cancer cells.
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INTRODUCTION

OF THE solid tumours, breast cancer is one of the most difficult to study cytogenetically beause of the poor growth of breast cancer cells *in vitro* and the frequent occurrence of cases with highly abnormal metaphases, which makes karyotyping almost impossible. Nevertheless, it is possible to identify a number of recurrent anomalies [1, 2]. Several chromosome losses, which are also seen as losses of heterozygosity, suggest that some events of tumour progression are determined by recessive genes, the deletion of one allele unmasking the remaining mutated allele.

To classify the tumours, we have taken advantage of the wide range of chromosome anomalies to differentiate cases with a few from those with many rearranged chromosomes, and also hypodiploid and near diploid from hyperploid cases. We studied chromosome banding by incorporation of 5-bromodeoxyuridine (BrdU) to investigate cell cycle progression [3–9], a method rarely used in cancer cytogenetics. To our knowledge, an anomaly of the cell cycle has been described in a single constitutional disease: Fanconi anaemia [10]. Data from transformed or cancerous cells are not more numerous, and their study is complicated by the possible anomalies in BrdU incorporation which may be related to unusual metabolism of BrdU [11]. One of the particularities of transformed or cancerous cells is their capacity to undergo many successive cycles, without pausing in G0 phase. This may give the false impression that they are cycling quickly. We would like to report that cells from mammary adenocarcinoma arrived in first metaphase *in vitro* have a slow cycle, especially because of a very long G2 phase.

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MATERIALS AND METHODS

The clinicopathological data of the 24 tumours studied are shown in Table 1. The chromosomal data of 6 cases has been reported [12]. All cultures, initiated after biopsy or puncture, were short-term (1–4 days), and BrdU (10 μg/ml) was added to the culture medium from 7 to 72 h before harvesting metaphases. Other cultures, without addition of BrdU, were always done to assess R-banded karyotypes.

The degree of BrdU incorporation was evaluated with a modified fluorescence plus Giemsa technique [9]. When possible, 50 metaphases were analysed for each experimental condition. According to the classification proposed by Couturier and Antoine [13], seven phases or subphases were distinguished: (i) G2, no modification of chromosome staining; (ii) late S (lS),