Dynamic coupling of ^{99m}Tc-MIBI efflux and apoptotic pathway activation in untreated breast cancer patients

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Abstract. Our previous studies showed that the efflux rate of technetium-99m methoxyisobutylisonitrile (MIBI) is directly correlated to P-glycoprotein (Pgp) levels in breast carcinoma. The aim of this study was to test whether the Pgp-dependent efflux of ^{99m}Tc-MIBI is related to the apoptotic pathway activation in breast carcinoma. Thirty-three untreated non-consecutive patients were intravenously injected with 740 MBq ^{99m}Tc-MIBI and serial images were obtained up to 4 h. The rate of efflux was determined by mono-exponential fitting of decay-corrected time-activity curves. Tumour specimens were then obtained at surgery and processed for the determination of the apoptotic index by in situ end-labelling of DNA fragments (Tunel). The rate of tumour cell proliferation was also determined using Ki67 monoclonal antibody. All breast carcinomas showed focal uptake of ^{99m}Tc-MIBI and the time to half clearance varied between 85 and 574 min. The apoptotic index ranged between 0.3% and 4.2%, whereas the rate of proliferation varied between 13% and 40%. We found a positive and significant correlation between the apoptotic index and the rate of proliferation (r=0.79, P<0.0001). The efflux rate of ^{99m}Tc-MIBI was directly and significantly correlated with the apoptotic index (r=0.74, P<0.0001) and with the rate of proliferation (r=0.58, P<0.001). After partial correlation analysis, only the apoptotic index showed a significant correlation with the efflux rate of ^{99m}Tc-MIBI (r=0.57, P<0.001). Our findings indicate that enhanced transport activity of Pgp is associated with increased activation of the apoptotic pathway, suggesting that inhibition of Pgp function with specific modulators may be effective in these patients. Furthermore, since mitochondria are central executioners of apoptosis and intracellular sites of ^{99m}Tc-MIBI sequestration, a model for the dynamic coupling of Pgp-dependent ^{99m}Tc-MIBI efflux and apoptotic pathway activation may be derived.

Keywords: ^{99m}Tc-MIBI efflux – Apoptosis – Breast cancer – Multidrug resistance

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Introduction

One of the most extensively studied mechanisms of drug resistance involves P-glycoprotein (Pgp), a member of the ATP-binding cassette (ABC) family of transporters, which is overexpressed in resistant tumours and actively extrudes a variety of compounds from cells [1, 2]. Since a number of anti-cancer drugs, including anthracycline, *Vinca* alkaloids, epipodophyllotoxins, actinomycin D and taxol, are recognised as substrates by Pgp, many chemotherapeutic regimens may become ineffective due to intrinsic or acquired resistance of tumour cells. Recently, the availability of radiolabelled substrates for Pgp raised the possibility of imaging Pgp function in cancer patients and identifying those who will become refractory to subsequent chemotherapy [3]. In particular, we have shown that the efflux rate of technetium-99m methoxyisobutylisonitrile (MIBI), a myocardial perfusion tracer and a Pgp substrate, is directly correlated with P-glycoprotein levels in untreated breast cancer patients [4] and that enhanced tracer efflux predicts lack of tumour response to neo-adjuvant therapy in patients with locally advanced breast cancer [5].

In an attempt to overcome the Pgp-dependent mechanism of multidrug resistance (MDR) in cancer patients, considerable efforts have been focussed on the development of modulators that can inhibit Pgp function through competitive binding or interaction with non-drug binding sites of the transporter [6, 7]. A broad range of compounds have been reported to reverse the MDR phenotype both in cellular and in animal models [7]. However, clinical trials with first-generation modulators have shown only limited success and considerable toxicity. Second-generation modulators, such as PSC833 and GG918, are currently being investigated in clinical settings [8] and novel, very potent and specific modulators have also been identified [9]. Despite the proven ability of such compounds to inhibit Pgp function both in vitro and in vivo, tumours can remain refractory to chemotherapy owing to the co-existence of other resistance mechanisms, such as alteration of the apoptotic pathway [10].

Apoptosis is an actively regulated cellular process leading to selective cell death. It may be triggered by several stimuli, such as radiation, drugs, toxins, deprivation of hormones or growth factors [11]. The morphological feature of apoptotic cell death is the condensation of chromatin and margination at the nuclear periphery, whereas the biochemical end point of the process is DNA fragmentation by endonucleases [12, 13]. Apoptosis is often increased in malignant tumours. In breast carcinoma, increased apoptosis is associated with a low degree of differentiation, tumour aneuploidy and poor prognosis [14, 15, 16]. During the last decade, it has become increasingly clear that regardless of distinct mechanisms of action, most anticancer agents exert their lethal effect by inducing apoptosis [17]. The inability of tumour cells to undergo apoptosis in response to chemotherapy may be responsible for treatment failure. Thus, in the presence of an altered apoptotic pathway, treatment may remain ineffective although the inhibition of Pgp by specific modulators may allow anticancer agents to accumulate in Pgp-overexpressing tumours.

The aim of this study was to determine the Pgp-dependent efflux of ^{99m}Tc-MIBI and to test the apoptotic pathway activation in untreated breast carcinoma.

Materials and methods

Patients. Thirty-three patients with histologically confirmed breast carcinoma (32 primary and 1 local recurrence) were studied. The mean age was 56±11 years. Tumours were classified according to the WHO nomenclature and included 25 infiltrative ductal, six lobular and two duct-lobular carcinomas. The tumour-nodes-metastasis (TNM) system was used for staging, and tumour size ranged between 1.2 and 7 cm. All patients gave informed consent prior to their inclusion in the study. No patient had received previous chemotherapy or preoperative local radiotherapy.

Scintigraphic study. Patients received 740 MBq of ^{99m}Tc-MIBI by i.v. injection in the arm contralateral to the lesion. Serial planar images were obtained using a gamma camera equipped with a low-energy general-purpose collimator and interfaced with a computer system (Digital, Maynard, Mass.). The patient lay prone on a scintimammography pad with a single breast dependent from the imaging pad. The detector, set for the lateral view, touched the patient's side. Dynamic data were acquired every minute for 15 min from the affected breast. Then, static 5-min images of both breasts were obtained at 0.5, 1, 2 and 4 h with the patient in the same position. Patients were carefully repositioned at each imaging time using external markers.

Regions of interest were drawn around each lesion in the frame of maximal tumour activity and then translated to all the other images. Decay-corrected time-activity curves were then generated from the selected areas and the efflux rates of ^{99m}Tc-MIBI were calculated using a mono-exponential fitting.

Determination of apoptotic index and rate of proliferation. Tumour specimens were obtained at surgery within 1 week of the scintigraphic study. Formalin-fixed paraffin-embedded tumour sections (5 µm thick) were obtained from the largest cross-sectional area of each tumour and processed for determination of the apoptotic index by in situ end-labelling of DNA fragments (Tunel) [18] using a commercially available kit (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy). Tumour sections were deparaffinated in xylene and rehydrated through passages in decreasing concentrations of ethanol. Then they were incubated with proteinase K (20 µg/ml) for 20 min at 22°C; thereafter, following washing with TBS and inactivation of endogenous peroxidase with 3% H_2O_2 in methanol, they were incubated with the labelling reaction mixture containing terminal deoxynucleotidyl transferase enzyme and biotinylated deoxynucleotides for 1.5 h at 37°C according to the manufacturer's instructions. The reaction was stopped with 0.5 *M* EDTA, pH 8 and revealed by the addition of peroxidase streptavidin conjugate and diaminobenzidine as chromogen. Finally, tumour sections were counterstained with 0.3% methyl green, dehydrated and mounted with a glass coverslip. Untreated and actinomycin D-treated HL60 promyelocytic leukaemia cells were provided by the manufacturer and used as negative and positive controls, respectively. The tumour sections were examined by light microscopy and divided into four to ten regions. A minimum of 100 tumour cells were counted in each region and the apoptotic index was determined as the mean percentage of positively stained tumour cells in a section.

The rate of tumour cell proliferation was determined on adjacent tumour sections using immunoperoxidase staining and Ki67 monoclonal antibody. This antibody recognises a nuclear antigen expressed in all phases of the cell cycle except G_0. The sections were examined by light microscopy as previously described [19] and the results were expressed as the percentage of proliferating tumour cells in a section.

Statistics. The efflux rate of ^{99m}Tc-MIBI was compared with the apoptotic index and the rate of proliferation using simple regression analysis and Pearson's coefficient of correlation. Owing to the significant correlation between the apoptotic index and the rate of proliferation, partial correlation analysis was also performed to test the correlation of the two variables with the effect of one variable controlled. Student's *t* test was used to assess differences between means. A probability value (*P*) of less than 0.05 was considered significant.

Table 1. Clinical data and pathological findings in 33 patients with untreated breast carcinoma

Patient no.	Age (yr)	Histology	Pathological stage	Size (cm)
1	74	Ductal	T3N0M0	>5
2	58	Ductal	T2N1M0	2.5
3	71	Ductal	T1N1M0	1.5
4	42	Ductal	T2N1M0	3
5	47	Ductal	T4N0M0	1.2
6	52	Ductal	T2N0M0	5
7	43	Ductal	T2N0M0	3
8	56	Ductal	T2N1M0	5
9	41	Ductal	T2N1M0	2.5
10	52	Ductal	T2N0M0	3
11	35	Ductal	T2N1M0	2.5
12	61	Ductal	T1N1M0	2
13	80	Ductal	T2N0M0	3
14	39	Lobular	T2N0M0	3
15	49	Ductal	T2N1M0	3
16	51	Ductal	T1N0M0	1.8
17	61	Ductal[a]	M0[b]	2
18	68	Ductal	T2N1M0	5
19	64	Ductal	T2NXM0[c]	4
20	62	Ductal	T1N0M0	2
21	62	Ductal	T2N0M0	5
22	69	Ductal	T2N0M0	2.2
23	50	Ductal	T1N0M0	2
24	56	Ductal	T1N0M0	1.3
25	55	Lobular	T2N1M0	4
26	63	Ductal	T2N1M0	2.8
27	65	Duct-Lob	T2N1M0	2.5
28	77	Duct-Lob	T4N1M0	7
29	57	Lobular	T2N0M0	2.5
30	47	Lobular	T2N1M0	4.8
31	60	Lobular	T1N0M0	1.2
32	47	Lobular	T1N1M0	2
33	48	Ductal	T2N1M0	4

Duct-Lob, Duct-lobular
[a] Local recurrence
[b] No distant metastasis at restaging
[c] Axillary dissection not performed

Table 2. Efflux rate of ^{99m}Tc-MIBI with the corresponding time to half clearance, apoptotic index and rate of proliferation in 33 patients with untreated breast cancer

Patient no.	Time to half clearance (min)	Efflux rate (min^{-1})	Apoptotic index (%)	Rate of proliferation (%)
1	85	0.00815	3.5	36
2	117	0.00592	2.0	28
3	132	0.00525	1.8	28
4	137	0.00506	1.7	31
5	146	0.00475	1.2	25
6	150	0.00462	4.2	40
7	168	0.00412	1.5	17
8	169	0.00410	1.5	27
9	176	0.00394	1.4	25
10	181	0.00383	2.0	31
11	199	0.00348	0.8	24
12	209	0.00332	0.8	23
13	221	0.00314	1.0	13
14	223	0.00311	0.5	21
15	223	0.00311	1.3	22
16	233	0.00297	1.2	25
17	243	0.00285	1.7	33
18	247	0.00281	1.7	30
19	252	0.00275	1.2	25
20	264	0.00262	1.2	29
21	274	0.00253	1.6	26
22	292	0.00237	1.0	18
23	317	0.00219	1.2	24
24	339	0.00204	0.6	21
25	343	0.00202	0.6	26
26	394	0.00176	0.4	18
27	397	0.00175	1.5	25
28	445	0.00156	0.3	26
29	471	0.00147	0.4	18
30	492	0.00141	0.5	16
31	501	0.00138	0.3	17
32	550	0.00126	0.9	19
33	574	0.00121	0.4	ND

[a] Not determined

Results

Table 1 summarises the clinical data and pathological findings of all patients included in the study. A high focal uptake of ^{99m}Tc-MIBI was found in all tumours.

The efflux rate determined on serial images of the lesion varied between 0.00121 and 0.00815 min^{-1}, which corresponded to a time to half clearance ranging between 85 and 574 min. Table 2 reports the efflux rate and the corresponding time to half clearance obtained in each patient.

The apoptotic index ranged between 0.3% and 4.2%, whereas the rate of proliferation varied between 13% and 40%. The apoptotic index and rate of proliferation of each tumour are reported in Table 2.

Simple regression analysis showed that the rate of proliferation and the apoptotic index were significantly correlated (r=0.79, P<0.0001) (Fig. 1), in agreement with the data reported by other authors [20, 21]. When we compared the efflux rate of ^{99m}Tc-MIBI with the apoptotic index, a strong, direct and significant correlation was found (r=0.74, P<0.0001) (Fig. 2). The efflux rate of ^{99m}Tc-MIBI was also correlated with the rate of proliferation, although with a lower coefficient of correlation (r=0.58, P<0.001).

Since the mechanisms of proliferation and apoptosis are known to be modulated in a coordinated manner [22], and due to the statistical correlation between rate of proliferation and apoptotic index in our study, we performed partial correlation analysis to determine the rela-

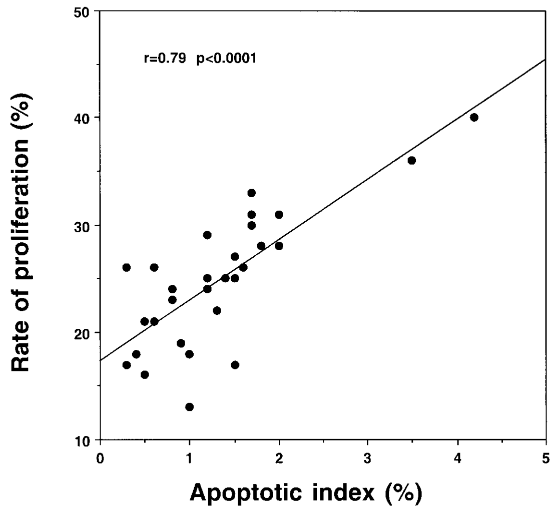

Fig. 1. Correlation between the rate of proliferation and apoptotic index measured in surgically excised breast carcinomas (Pearson's coefficient of correlation r=0.79; P<0.0001)

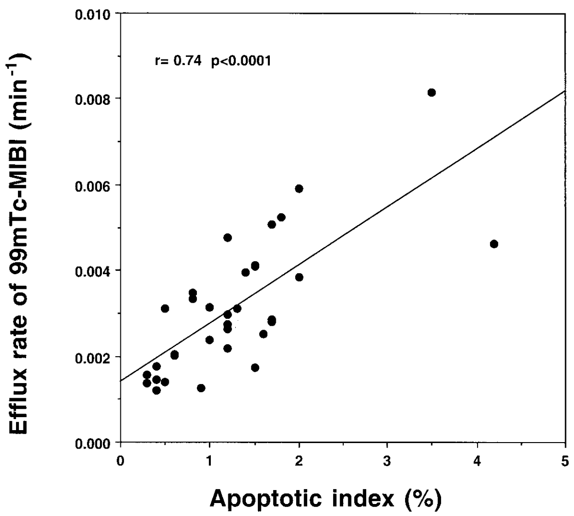

Fig. 2. Correlation between the efflux rate of ^{99m}Tc-MIBI determined in vivo on serial scintigraphic images and the apoptotic index measured in surgically excised breast carcinomas (Pearson's coefficient of correlation r=0.74; P<0.0001)

Table 3. Partial correlation analysis: the correlation of the two variables, namely rate of proliferation and apoptotic index, with ^{99m}Tc-MIBI efflux was tested

	r	*P*
Rate of proliferation	–0.009	0.96
Apoptotic index	0.57	0.0007

tive dependence of the efflux rate of ^{99m}Tc-MIBI on each of the two variables. After partial correlation analysis only the apoptotic index was significantly correlated with the tracer efflux (r=0.57, P=0.0007) (Table 3).

Using a cut-off value for tracer clearance of 204 min derived by our previous studies [4], breast tumours were then divided into two groups: those with fast ^{99m}Tc-MIBI clearance ($T_{1/2}$<204 min) and those with slow tracer clearance ($T_{1/2}$>204 min). The mean value of the apoptotic index was twofold higher in tumours with fast ^{99m}Tc-MIBI clearance than in tumours with slow tracer clearance, with a statistically significant difference between the two groups (mean±SD, 1.96±1.00 vs 0.92±0.47, P<0.001). The apoptotic index did not show any relationship with lymph node status and did not correlate with age and tumour size. On the other hand, there was a significant relationship with histology as invasive ductal carcinomas showed a higher apoptotic index than did invasive lobular carcinomas (mean±SD 1.47±0.84, n=25 vs 0.53±0.20, n=6, P=0.01). Although the number of invasive lobular carcinomas in our study was limited, this finding confirmed previous reports by other authors [23].

The rate of proliferation was significantly different in tumours with fast and slow ^{99m}Tc-MIBI clearance (mean±SD, 28±6 vs 22±5, P<0.01) whereas it did not correlate with age, tumour size or lymph node status.

Discussion

Our study shows that an enhanced tumour efflux of ^{99m}Tc-MIBI determined in vivo in untreated breast cancer patients is associated with an increased activation of the apoptotic pathway assessed in vitro on surgically excised tumours. The direct and significant correlation found between the tracer efflux and the apoptotic cell fraction indicates that the functional assessment of Pgp status by ^{99m}Tc-MIBI scan may provide selection criteria with which to identify tumours with high Pgp levels and an activated apoptotic pathway. In agreement with our previous studies, which defined a cut-off value for clearance of 204 min [4], one-third of untreated breast carcinomas showed a fast tumour clearance of ^{99m}Tc-MIBI ($T_{1/2}$<204 min), indicating the presence of high levels of Pgp. These tumours showed a twofold higher apoptotic index as compared with that found in tumours with slow tracer clearance ($T_{1/2}$>204 min).

Recently, potent inhibitors of Pgp functions have been developed and tested in both experimental and clinical settings [7, 8].However, only limited success has been observed in clinical trials involving a wide range of malignancies, primarily lymphoma, leukaemia and multiple myeloma [7, 8]. Many factors may affect the clinical modulation of MDR, including the inadequate levels or low binding specificity of Pgp inhibitors at the tumour site and the coexistence of multiple resistance mechanisms, such as an altered control of apoptosis [10]. Our study shows that patients selected on the basis of a rapid ^{99m}Tc-MIBI efflux do have tumours with an activated apoptosis and that they will potentially benefit from treat-

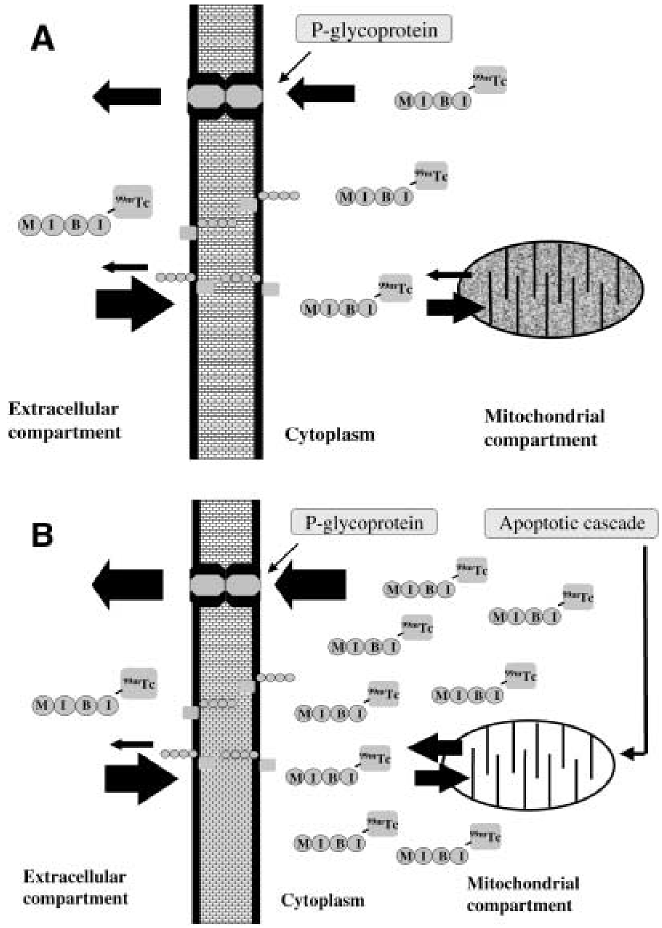

Fig. 3A, B. Schematic model of a possible dynamic coupling between the Pgp-mediated efflux of ^{99m}Tc-MIBI and the apoptotic pathway activation. **A** The uptake of ^{99m}Tc-MIBI from the extracellular compartment to the cytoplasm and its reversible accumulation within mitochondria are driven by the electronegative plasma membrane and mitochondrial membrane potentials. In resistant tumours, ^{99m}Tc-MIBI efflux is enhanced by the energy-dependent activity of P-glycoprotein, which is responsible for the active outward transport of the tracer. **B** The integration of death signals at mitochondrial levels leads to permeabilisation of mitochondrial membrane and disruption of mitochondrial membrane potentials. This may result in a reduced ability of mitochondria to retain or accumulate ^{99m}Tc-MIBI. In these circumstances, since ^{99m}Tc-MIBI is not sequestered within mitochondria, its binding to Pgp may be favoured and the outward tracer transport may be enhanced

ment with Pgp modulators. The inhibition of Pgp function would result in an increase in the intracellular concentration of MDR drugs, which in turn might exert their lethal effect by inducing apoptosis through an effective pathway.

The mechanism underlying the association of Pgp-mediated ^{99m}Tc-MIBI efflux and the apoptotic pathway activation is presently unknown. However, a clue emerges from consideration of the uptake and release modalities of ^{99m}Tc-MIBI in resistant malignant cells (Fig. 3). Many studies have shown that ^{99m}Tc-MIBI enters the cell in response to large negative plasma membrane and mitochondrial inner membrane potentials and accumulates reversibly within mitochondria of both malignant and normal cells [24, 25]. Mitochondria are also reported to be the central executioners of apoptosis [26]. They integrate death signals through Bcl-2 family members and coordinate caspase activation through the release of cytochrome c from the mitochondrial compartment [27]. There is also increasing evidence that mitochondrial membrane permeabilisation and the consequent dissipation of the mitochondrial transmembrane potentials is a critical step in the apoptotic cascade and that both events precede the appearance of the classical signs of cell death [28]. Faster ^{99m}Tc-MIBI release from mitochondria to the cytoplasm may be caused by mitochondrial membrane permeabilisation. Alternatively, decreased ^{99m}Tc-MIBI accumulation within mitochondria may be due to disruption of potentials. On the basis of these considerations, it is conceivable that in the early phases of apoptosis, when energy-dependent processes may still occur, the Pgp-mediated transport of ^{99m}Tc-MIBI from the cytoplasm to the extracellular compartment is enhanced by the reduced ability of the mitochondrial compartment to retain or accumulate ^{99m}Tc-MIBI. Therefore, our findings may reflect a dynamic coupling between the Pgp-mediated efflux of ^{99m}Tc-MIBI from tumours and the apoptotic pathway activation.

In ^{99m}Tc-MIBI-positive breast carcinomas, the percentage of apoptotic cells varied between 0.3% and 4.2%, and this may appear a very limited fraction of cells to cause variation in ^{99m}Tc-MIBI kinetics detectable by region of interest analysis over a whole lesion. However, detection of apoptotic cells by staining of DNA fragments or by morphological changes identifies only the fraction of cells in the final phases of the process. The percentage of tumour cells undergoing the apoptotic pre-degradation steps is probably higher since it has been reported that identifiable apoptotic cells may account for only 10% of the cells entering the pathway for programmed death [29].

Taken together, our findings show that the kinetic analysis of ^{99m}Tc-MIBI efflux in untreated breast cancer patients may provide information on the functional status of Pgp and on the activation status of the apoptosis machinery. Therefore, ^{99m}Tc-MIBI efflux kinetics may be proposed as selection criteria with which to identify patients who are candidates for treatment with Pgp modulators. Although further clinical studies are needed, the use of specific Pgp modulators is expected to enhance the tumour response rate only in patients with high Pgp levels and an effective apoptotic pathway.

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