ANTICANCER RESEARCH *25*: 3963-3966 (2005)

Alteration in Platelet Function in Patients with Early Breast Cancer

G. McDOWELL[1,2], I. TEMPLE[3], C. LI[4], C.C. KIRWAN[3], N.J. BUNDRED[3], C.N. McCOLLUM[3], I.E. BURTON[5], S. KUMAR[4], G.J. BYRNE[3]

[1]Department of Clinical Biochemistry, Manchester Royal Infirmary, Oxford Road, Manchester, M13 9WL; [2]Department of Clinical Biochemistry, [3]Department of Surgery and [5]Department of Haematology, South Manchester University Hospital NHS Trust, Wythenshawe Hospital, Southmoor Road, Manchester, M23 9LT; [4]Department of Pathology, Medical School, University of Manchester, Manchester M13 9PT, U.K.

Abstract. *The aim of this study was to examine our hypothesis that platelets of patients with breast cancer were functionally altered compared to healthy controls. The results have shown that the platelets from women with early breast cancer released significantly more vascular endothelial growth factor (VEGF) when stimulated with thrombin, tissue factor, clotting, or over a period of time. Similarly, release of thrombospondin (TSP) with thrombin and tissue factor was higher, but failed to reach a significant level. Thus, the observed differences in platelet response support our hypothesis, but warrant further work to determine the reason underlying the observed difference and potential clinical relevance of our findings.*

The requirement for platelets in cancer metastasis was recognised about 30 years ago and it is now recognised that platelets are an integral part of the microthrombus that is thought to promote the arrest and lodgement of circulating tumour cells (1-3). Indeed the formation of metastasis is reduced in the absence of platelets (4), but the reason for this is not clearly understood. Platelet-cell interactions mediated *via* platelet-selectin (P-selectin) have been suggested and tumours in P-selectin-deficient mice have reduced metastatic potential (4). It has also been suggested that tumour cell-platelet aggregates protect tumour cells from immune-mediated cytolysis (1, 5).

Correspondence to: Dr. Garry McDowell, Department of Clinical Biochemistry, Manchester Royal Infirmary, Oxford Road, Manchester M13 GWL, U.K. Tel: 0044 161 276 6728, Fax: 0044 161 276 4586, e-mail: garry.mcdowell@manchester.a.uk

Key Words: Platelets, breast cancer, VEGF, thrombospondin, tissue factor.

Platelets are numerous in the circulation and contain large stores of factors known to be essential for the development of tumour angiogenesis. Thus, platelets may have a role in the progression of metastasis. Vascular endothelial growth factor (VEGF) is one such protein that is stored in large quantities in platelet α-granules (6) and elevated circulating levels have been observed in patients with malignancy including breast cancer (7-9). VEGF is pro-angiogenic and it has been shown that VEGF secreted by megakaryocytes may contribute to proliferation of vascular endothelial cells (10). Thrombospondin (TSP-1) is another platelet α-granule protein with diverse physiological roles and increased plasma levels have been noted in patients with breast cancer (11).

We hypothesized that platelets are functionally altered in women with breast cancer to promote the development of metastasis. The aim of this investigation was, therefore, to investigate the release of TSP-1 and VEGF from stimulated platelets in women with early breast cancer.

Methods

Patients with early breast cancer. Eleven patients undergoing surgery for early breast cancer were studied. Surgically the patients were classified as wide local excision with axillary node clearance (5), wide local excision (4), bilateral mastectomy (1) and ultrasound-guided diagnostic biopsy (1). Haematoxylin and eosin-stained sections were examined for grade of tumour, vascular invasion, tumour load, lymph node status and oestrogen and progesterone receptor status by a consultant histopathologist, who was blinded to the VEGF or TSP-1 results. The patient details are summarised in Table I.

Control group. The control group consisted of patients undergoing surgery for benign breast lumps (2 fibroadenomas, 2 scars, 1 cyst and 1 benign microcalcification) or cosmetic reasons (1 nipple eversion) and 4 healthy members of staff.

Table I. *Histological details of patients with early breast cancer.*

	Tumour type	Histological grade	Vascular invasion	Lymph node status	ER	PR
1	Infiltrating ductal	3	No	Neg	Neg	Neg
2	Infiltrating ductal	2	No	Pos (1/20)	Pos (100%)	Pos (50%)
3	Infiltrating ductal	2	Yes	Pos (1/19)	Pos (100%)	Pos (70%)
4	Infiltrating ductal	3	Yes	Neg	Pos (100%)	Pos (100%)
5	DCIS	High	No	Neg	Pos (100%)	Pos Occasional
6	DCIS	High	No	Neg	Neg	Neg
7	Infiltrating ductal	2	No	Neg	Pos (40%)	Pos (70%)
8	DCIS	High	No	Neg	?	?
9	Infiltrating ductal	1	No	Neg	Pos (100%)	Pos (60%)
10	Lobar carcinoma Grade1	2	No	Neg	Pos (100%)	Pos (70%)
11	Infiltrating ductal	2	No	Neg	Pos (100%)	Pos (70%)

ER=oestrogen receptor and PR=progesterone receptor status.

Sample collection and processing. All subjects gave written informed consent. Samples were drawn from an antecubital fossa vein *via* a 19G needle and syringe prior to surgery. Blood was collected into CTAD (n=1), citrate (n=4), EDTA (for full blood count) (n=1) and finally a plain clotting (serum) (n=1) tube. Three of the citrate samples were centrifuged at room temperature for 10 min at 180 xg to obtain platelet rich plasma (PRP). The top 1 ml of plasma from each citrate tube was combined and mixed by gentle inversion to yield 3 ml of PRP. Three aliquots of PRP (2x 1 ml and 1x 0.6 ml) were taken and the remaining volume used to assess platelet count. Full blood count and platelet count were measured on the Advia Clinical Haematology analyser.

Processing of PRP samples

Stimulation of platelet rich plasma by thrombin: Thrombin (lyophilized bovine thrombin reconstituted with 5 ml distilled water to give approximately 100 units/ml (Dade Behring, Marburg, Germany) (40 μl) was added to 1 ml of PRP. The sample was mixed by inversion and incubated for 15 min at room temperature.

Stimulation of platelet rich plasma by recombinant tissue factor: Pre-warmed (37˚C) recombinant tissue factor (lyophilized human tissue factor (Innovin, Dade Behring) reconstituted with 10 ml of distilled water) (1 ml) was added to 0.6 ml of PRP. The sample was mixed and incubated for 15 min at room temperature.

Unstimulated platelet rich plasma and the effect of clotting: A 1-ml aliquot of PRP was left at room temperature for 15 min to control for the effect of the above incubation times. To investigate the effect of clotting on the release or VEGF and TSP-1, a plain sample was allowed to clot before separation of the serum.

Analysis of VEGF and TSP-1. At the end of the above incubations, the three PRP samples, the CTAD and serum samples were centrifuged for 15 min at 3500 xg. The supernatant from each sample was separated and centrifuged again for 15 min at room temperature and 3500 xg to yield platelet depleted plasma. Each of the 5 sample aliquots were stored at –80˚C prior to analysis.

VEGF was assayed by commercial ELISA according to the manufacturer's instructions (R and D systems, Oxon, UK). In duplicate, a sample (100 μl) diluted with 100 μl of assay diluent was incubated with the capture antibody for 2 h at room temperature. Following washing steps (x3), 200 μl of detection antibody conjugate was added and the assay incubated for 2 h at room temperature. The plate was again washed (x3) and the detection substrate added and incubated for 25 min at room temperature. Stop solution (25 μl) was added and the optical density read at 450nm.

TSP-1 was measured as previously described (11). Briefly, 50 μl of sample, in duplicate, was added to 200 μl of ^{125}I -labelled TSP (0.5 ng/tube) and 200 μl of rabbit anti-TSP antibody (1:10000 dilution in assay buffer) and incubated at 4˚C overnight. Then 300 μl of Sac-cel (anti-rabbit) second antibody was added and the assay incubated for 30 min at room temperature, followed by addition of 3 ml of wash solution and centrifugation for 20 min at 1500 xg and 8˚C. The supernatant was decanted and the precipitate containing the bound fraction counted for 120 sec on an LKB 1260 multi-well counter (Wallac, Milton Keynes, UK).

Calculation of results. The results were corrected for dilution associated with adding tissue factor or thrombin. By subtracting the concentration of VEGF or TSP-1 in the unstimulated PRP from the concentration in the stimulated PRP samples, it is theoretically possible to determine the increase in concentration of VEGF or TSP-1 that has been released due to thrombin or tissue factor stimulation. This can then be divided by the platelet count of the PRP to determine a theoretical value for the VEGF or TSP-1 that has been released per platelet. In a similar fashion, it is possible to theoretically determine the amount of VEGF or TSP-1 that has

Table II. *VEGF released from platelets in controls and patients with early breast cancer*

	VEGF released due to thrombin stimulation. (pg/10^6 plts)	VEGF release due to tissue factor stimulation (pg/10^6 plts)	VEGF release due to time (pg/10^6 plts)	VEGF release due to clotting (pg/10^6 plts)
Control group	0.476 [0.00-5.56]	0.510 [0.09-4.42]	0.017 [0.02-0.48]	0.217 [0.04-0.56]
EBC group	1.423 [0.52-5.58]*	1.045 [0.48-6.45]**	0.104 [0.05-0.32]***	0.368 [0.15-1.75]

VEGF results expressed as median [range]. Results are expressed as VEGF released per 10^6 platelets in the control group and early breast cancer group following stimulation with thrombin or tissue factor, unstimulated degranulation and clotting. *$p=0.017$; **$p=0.025$; ***$p=0.01$ (Mann-Whitney *U*-test compared to controls).

Table III. *TSP-1 released from platelets in controls and patients with early breast cancer*

	VEGF released due to thrombin stimulation. (pg/10^6 plts)	VEGF release due to tissue factor stimulation (pg/10^6 plts)	VEGF release due to time (pg/10^6 plts)	VEGF release due to clotting (pg/10^6 plts)
Control group	126 [-36-294]	30.1 [2.34-172]	70 [-31-114]	22 [-63-78]
EBC group	218 [-156-525]	45 [-135-259]	30 [-59-109]	37 [-36-82]

TSP-1 results expressed as median [range]. Results are expressed as TSP-1 released per 10^6 platelets in the controls and early breast cancer group following stimulation with thrombin or tissue factor, unstimulated degranulation and clotting.

been released per platelet due to the 45 min that the unstimulated PRP has been left at room temperature. Finally, it is possible to determine the amount of VEGF or TSP-1 released by clotting. The equations are as presented:

$$\text{TSP or VEGF released due to thrombin} = \frac{\text{Thrombin stimulated PRP} - \text{Unstimulated PRP}}{\text{PRP platelet count}}$$

$$\text{TSP or VEGF released due to time} = \frac{\text{Unstimulated PRP}}{\text{PRP platelet count}} - \frac{\text{Plasma}}{\text{FBC platelet count}}$$

$$\text{TSP or VEGF released due to clotting} = \frac{\text{(Serum} - \text{plasma) x (1} - \text{haematocrit)}}{\text{FBC platelet count}}$$

These corrections have been validated and published previously (12).

Statistical analysis. As none of the results were normally distributed non-parametric statistics were used throughout. A Mann-Whitney *U*-test was used to compare independent variables between groups.

Results

The median ages of the control and early breast cancer patients were 49 (31-64) and 58 (48-70) years, respectively ($p=0.002$). The VEGF results are shown in Table II. The results show a significant difference in the amount of VEGF released per platelet between early breast cancer and the control group for thrombin stimulation ($p=0.017$), tissue factor stimulation ($p=0.025$) and unstimulated degranulation ($p=0.010$). There was no significant difference between the two groups for VEGF release per platelet upon clotting. The TSP-1 results are shown in Table III. There were no significant differences in TSP-1 release per platelet following thrombin, tissue factor, unstimulated degranulation or clotting.

Discussion

The aim of this study was to ascertain whether the platelets of patients with early breast cancer were functionally altered compared to normal controls. Platelet α-granules are a rich source of compounds that are thought to be involved in many

of the processes essential to the formation of metastases (1). The results of this study showed that the platelets from women with early breast cancer release significantly more VEGF when stimulated with thrombin, tissue factor or time. Adams *et al.* (12) investigated the amount of VEGF released during clotting as a measure of VEGF in platelets, suggesting that clotting would release all platelet α-granule VEGF. The results of our investigation calls into question the methodology of Adams *et al.* Our results demonstrated that, whilst there was a small difference between the early breast cancer group and controls, this did not reach statistical significance. Either clotting does not release all α-granule VEGF or that VEGF is becoming trapped in the protein clot are potential explanations for this finding.

Salven *et al.* (13) investigated the release of VEGF from platelets following cell lysis by repeated osmotic shock with sterile water and repeated freeze thaw cycles in a heterogeneous group of patients. The results observed were similar to those observed in this study.

Thrombospondin is a high molecular weight glycoprotein which is synthesised and secreted by a number of cells including platelets. In platelets TSP is also stored in α-granules (14). Circulating plasma levels of TSP have been shown to be increased in patients with breast cancer (11) and TSP is also thought to be important in cancer metastases. TSP has both angiogenic and anti-angiogenic properties in a dose-dependent manner. The results of this investigation, however, have not shown a significant difference between the two groups in TSP released following stimulation of platelets with thrombin, tissue factor, time or clotting, suggesting that the elevated circulating plasma levels may arise from other sources.

In conclusion, a differential response in the release of VEGF and TSP following platelet stimulation with thrombin and tissue factor was observed. The reason for this difference and the potential pathophysiological significance of these observations remain unknown. With regard to possible implication of our findings, our unpublished study indicates that the products of platelets activation play a role in post-radiotherapy-induced fibrosis in breast cancer patients. Folkman and his colleagures, some years ago, recognized a cancer-specific role for the platelets and suggested that differences in the release of pro- and anti-angiogenic factors by this cell modulated the angiogenic switch that controls tumour growth and spread (15). Similarly, it has been proposed that platelet-associated angiogenesis is important in many neuro-cardiovascular diseases (16).

References

1 Trikha M and Nakada MT: Platelets and cancer: implications for antiangiogenic therapy. Semin Thrombo Haemostas *28*: 39-44, 2002.

2 Hejna M, Raderer M and Zielinski CC: Inhibition of metastases by anticoagulants. J Natl Cancer Inst *91*: 22-36, 1999.

3 Tang DG and Honn KV: Adhesion molecules and tumour metastasis: an update. Invasion Metastasis *14*: 109-122, 1994.

4 Young K, Borsig L, Nissi M *et al*: P-selectin deficiency attenuates tumour growth and metastasis. Proc Natl Acad Sci *95*: 9325-9330, 1998.

5 Gasparini G, Toi M, Gion M *et al*: Prognostic significance of vascular endothelial growth factor in node negative breast carcinoma. J Natl Cancer Inst *89*: 139-147, 1997.

6 Ferrara N and Davis-Smyth T: The biology of vascular endothelial growth factor. Endocrine Rev *18*: 4-21, 1997.

7 Ferrari G and Scagliotti GV: Serum vascular endothelial growth factor levels in non-small cell lung carcinoma. Eur J Cancer *32A*: 2369-2370, 1996.

8 Dirix LY, Vermeulen PB, Pawinski A *et al*: Elevated levels of angiogenic cytokines basic fibroblast growth factor and vascular endothelial growth factor in sera of cancer patients. Br J Cancer *76*: 238-243, 1997.

9 Oehler MK and Carrier H: Diagnostic value of serum VEGF in women with ovarian tumours. Anticancer Res *19*: 2519-2522, 1999.

10 Mohle R, Green D, Moore MAS, Nachman RL and Rafii S: Constitutive production and thrombin induced release of vascular endothelial growth factor by human megakaryocytes and platelets. Proc Natl Acad Sci *94*: 663-668, 1997.

11 Hayden KE, Tetlow L and Byrne G: Radioimmunoassay for the measurement of thrombospondin in plasma and breast cyst fluid: validation and clinical application. Ann Clin Biochem *37*: 319-325, 2000.

12 Adams JA, Carder PJ, Downey S *et al*: Vascular endothelial growth factor in breast cancer: comparison of plasma, serum and tissue VEGF and microvessel density and effects of tamoxifen. Cancer Res *60*: 2898-2905, 2000.

13 Salven P, Orpana A and Joensuu H: Leukocytes and platelets of patients with cancer contain high levels of vascular endothelial growth factor. Clin Cancer Res *5*: 487-491, 1999.

14 Sargiannidou I, Zhou J and Tuszynski GP: The role of thrombospondin-1 in tumour progression. Exp Biol Med *226*: 726-733, 2001.

15 Pinedo HM, Verheul HMW, D'Amato RJ and Folkman J: Involvement of platelets in tumour angiogenesis? Lancet *352*: 1775-1777, 1998.

16 Blann AD: Angiogenesis and platelets: the clot thickens further. Cardiovasc Res *63*: 192-193, 2004.

Received September 9, 2005
Accepted September 21, 2005