Monocyte activation in patients with non-seminomatous germ cell tumour of the testis before and after tumour eradication

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Abstract

Aims—To investigate the kinetics of normalisation of monocyte oxidative activity following tumour eradication.

Methods—Whole blood lucigenin enhanced chemiluminescence was studied in patients with non-seminomatous germ cell tumours. Group 1 comprised 14 patients who had been “cured” of their cancer (the term “cured” as used in this report denotes a relapse free period of at least three years). Group 2 comprised 15 patients who were followed from diagnosis to up to two years after the start of treatment.

Results—Lucigenin enhanced chemiluminescence of whole blood in the “cured” patients was similar to that of controls and lower than that in patients who had not yet received chemotherapy (group 2). After treatment, chemiluminescence decreased slowly and did not normalise until 18 months after the start of treatment. Tumour necrosis factor α (TNF α) concentrations were normal in “cured” patients but were raised in those who had not yet received treatment. TNF α was normalised 12 months after start of treatment. Alpha-fetoprotein concentrations were raised in most patients but normalised rapidly after tumour eradication.

Conclusions—The activity of blood monocytes, as measured by whole blood lucigenin enhanced chemiluminescence, is increased in cancer. This activity may be a consequence of the presence of tumour cells. Immunocompetent cells remain active for over a year after eradication of the tumour.

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(TNF α), a cytotoxic molecule secreted by activated monocytes, in addition to its cytotoxic activity,^{13,14} also has broad immunomodulating effects.¹⁵⁻¹⁸ Earlier studies have shown that the chemiluminescence activity of whole blood is increased in patients with cancer,^{11,12} suggesting that monocyte activity is increased in these patients. The increased activity, however, did not seem to be related to cancer spread. In fact, there was a tendency towards lower monocyte activity in patients with metastases. The cause of this increased monocyte activity in patients with cancer is not clear and could either be the result of a genetically determined increase in activity in these patients, or a consequence of monocyte activation by products generated by the tumour itself or the principle cells involved in the host response.¹⁹ In an attempt to examine this further we studied whole blood chemiluminescence in patients with non-seminomatous germ cell tumours of the testis. The patient cohort was divided into two groups: those with no signs of relapse at least three years after treatment finished (group 1) and those who were followed from diagnosis to up to two years after treatment started. TNF α was measured as another marker of monocyte activity^{19,20} and α -fetoprotein (AFP) as a marker of cancer cell growth.²¹⁻²³

Methods

All patients underwent orchiectomy and the diagnosis of non-seminomatous germ cell tumour of the testis was confirmed by histopathology. After surgery, all of the patients underwent chest x ray and a computed tomography (CT) scan of the pelvis and abdomen. The serum concentrations of AFP and β human chorionic gonadotropin (β -HCG) were measured before surgery and at each follow up visit. Serum concentrations of AFP and β -HCG were regarded as raised if they were above 15 μ g/l and 5 U/l, respectively. Tumour markers were assessed before the beginning of each chemotherapy cycle and every four to eight weeks on follow up. Radiological examinations were carried out at two to four month intervals. In patients who underwent chemotherapy a complete response was defined as the resolution of all metastatic lesions and normalisation of tumour markers. The definition “cured” as used in this report refers to a relapse free period of at least three years. Relapse/progressive disease was defined as the appearance of new metastatic lesions and/or an increase in AFP and/or β -HCG concentrations.

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Defence against cancer spread depends on a variety of reactions, including that of phagocytic cells.¹⁻⁴ The mechanisms by which these cells eradicate cancer cells are probably analogous to the way microbes are killed—the generation of cytotoxic free radicals derived from oxygen,^{5,6} and the production and release of cytotoxic molecules.¹⁷ In whole blood the production of these oxygen radicals by monocytes can be measured by lucigenin enhanced chemiluminescence⁸⁻¹⁰; previous studies indicated that lucigenin enhanced chemiluminescence reflects the activity of blood monocytes.^{11,12} Tumour necrosis factor α

Figure 1 Whole blood lucigenin enhanced chemiluminescence in controls and patients with non-seminomatous germ cell tumour of the testis. The *p* values given in the figure were calculated using the Mann-Whitney U-test.

Chemotherapy comprised bleomycin, etoposide and cis-platinum. The following doses were used: cis-platinum (Platinol, Bristol-Myers) 20 mg/m² for five days; etoposide (Vepesid, Bristol-Myers) 100 mg/m² for five days; and bleomycin (Bleomycin, Lundbeck) 15 mg on days 1, 5 and 16. The patients underwent four cycles of this chemotherapy regimen, starting every three weeks.

Group 1 comprised 14 patients "cured" of their disease with an average age of 35 years (range 25–49 years). Group 2 comprised 15 patients with non-seminomatous germ cell tumour of the testis with an average age of 30 years (range 18–51 years). Nine of the 15 patients were followed for two years, four patients were followed while receiving chemotherapy (zero to three months) and two patients during the follow up period (three months to two years). Seventeen healthy laboratory employees (11 women and six men) served as controls. Their average age was 35 years (range 30–52 years). Fourteen healthy male subjects matched for age served as controls in the TNF α assay.

Blood was taken into tubes containing heparin before each chemotherapy cycle—that is, every third week for nine weeks and thereafter every four to eight weeks up to 24 months. The values during the follow up period are given as the median values for each patient during the following periods: three to six months; seven to 12 months; 13 to 18 months; and 19 to 24 months. Leucocyte counts, differentials and haemoglobin concentrations were measured on a Technicon H 1 blood cell analyser.

Figure 2 Kinetics of lucigenin enhanced chemiluminescence of whole blood. As indicated in the figure, chemiluminescence production decreased 13–18 months after the initiation of chemotherapy. Data are given as medians and 25–75 percentiles. ** *p* < 0.01.

CHEMILUMINESCENCE

Whole blood chemiluminescence was detected as described previously¹¹ using a modification of the method described by Tono-Oka *et al.*²⁴ Briefly, 50 μ l blood was mixed with 400 μ l Gey's buffer in a measuring vial and preincubated at 37°C for five minutes. Then, 100 μ l lucigenin (0.5 g/l) was added and the sample incubated for another five minutes. The vial was placed in a measuring chamber of Biocounter M2010 (Lumac B.V., The Netherlands) and 100 μ l serum opsonised zymosan was added. Chemiluminescence was recorded continuously. To adjust for chemiluminescence contributed by polymorphonuclear (PMN) cells in the blood, the results are expressed as relative light units (RLUs)/10⁶ PMN from the peak of the curve.

TNF α ASSAY

Serum TNF α was assayed using an enzyme linked immunosorbent assay (EASIA-Medgenix, Fleurus, Belgium). The protocol recommended by the manufacturer was followed.

STATISTICS

The Mann-Whitney U test and Wilcoxon test were used to evaluate differences between the patient groups and paired samples, respectively. Calculations were carried out on a PC using STATISTICA/W (StatSoft, USA) software.

Results

CHEMILUMINESCENCE OF WHOLE BLOOD

As shown in fig 1, lucigenin enhanced chemiluminescence in whole blood was significantly higher in patients with cancer who had not yet received chemotherapy than in controls and "cured" patients (*p* < 0.01 and *p* < 0.001, respectively).

Figure 2 shows the kinetics of normalisation of whole blood chemiluminescence. Lucigenin enhanced chemiluminescence did not decrease significantly until 13 to 18 months after the initiation of chemotherapy (*p* < 0.001, Mann-Whitney U test; *p* < 0.01, Wilcoxon's matched pair test).

Figure 3 TNF α serum concentrations. The *p* values given in the figure were calculated using the Mann–Whitney U-test.

SERUM CONCENTRATIONS OF TNF α
As shown in fig 3, TNF α concentrations were significantly higher before chemotherapy than in controls ($p<0.001$) or “cured” patients ($p<0.05$). A significant decrease in TNF α serum concentrations was observed 12 months after the first cycle of chemotherapy ($p<0.01$, Mann–Whitney U test) (fig 4).

SERUM CONCENTRATIONS OF AFP AND β -HCG
AFP and β -HCG are regularly used to diagnose and monitor non-seminomatous germ cell tumour of the testis. As shown in table 1, there were no significant differences between the serum concentrations of AFP before and after chemotherapy. In several patients with abnormal concentrations of AFP, the median value before treatment was raised and the range was very large. The pre-treatment serum concentrations of β -HCG in these patients were normal (data not shown).

BLOOD CELL COUNTS
The blood cell counts of patients with non-seminomatous germ cell tumour of the testis

Table 1 Alpha fetoprotein (AFP) concentrations in patients with non-seminomatous germ cell tumour of the testis

	<i>n</i>	AFP concentration (μ g/l)	
		Median	Range
Chemotherapy			
Before	10	15	4.4–1857
During	10	4.5	4.4–72
Follow up (months)			
3–6	8	4.5	4.1–6
7–12	11	4.6	4.4–8.5
13–18	9	5.9	4.4–7.5

n = number of patients.

Figure 4 Follow up of serum concentrations of TNF α . Data are given as medians and 25–75 percentiles. Significant differences were evaluated using the Mann–Whitney U-test. ** $p<0.01$.

before treatment, after 18 months of follow up, in “cured” patients, and in controls are shown in table 2. The neutrophil counts were significantly higher before chemotherapy ($p<0.05$) compared with those recorded 18 months later. Blood lymphocyte counts were significantly lower ($p<0.01$) before treatment when compared with controls.

Discussion
The results presented here confirm previous observations of increased lucigenin enhanced chemiluminescence in whole blood of patients with cancer.^{11 12} The data clearly indicate that this production is related to the cancer per se, as in the group of patients who were successfully “cured” of their cancer the activity was significantly lower and similar to the production of healthy subjects. Moreover, production was also normalised in the patients followed during and after treatment.
Two cell types contribute to lucigenin enhanced chemiluminescence in whole blood, PMN and monocytes. To eliminate the contribution from PMN, the results were adjusted for the number of PMN in the blood by being expressed as RLU/ 10^6 PMN. Therefore, any chemiluminescence observed is produced by monocytes or as a result of the altered activity of PMN neutrophils. However, our earlier studies indicated that the major contribution was from monocytes.¹² The reason for this is not quite clear, although purified monocytes produce relatively more chemiluminescence when treated with lucigenin than neutrophils. It has also been suggested that monocytes produce unique oxidative intermediates other than O_2^- , which react specifically with lucigenin.⁹ The increase in chemiluminescence observed in patients with cancer, therefore, is probably the result of increased monocyte activity. Indeed, in a previous report¹² we showed that monocytes exist in different subpopulations in blood with different oxidative activities and that the monocyte subpopulation with the highest activity is over-represented in patients with cancer. We also found that TNF α concentrations were increased in patients with cancer, which, as monocytes are probably the major producers

Table 2 Leucocyte counts in controls and patients with non-seminomatous germ cell tumour of the testis

	n	Neutrophils (10 ⁹ /l)	Lymphocytes (10 ⁹ /l)	Monocytes (10 ⁹ /l)	Eosinophils (10 ⁹ /l)
Before treatment	11	4.0* (0.8–8.5)	1.23† (0.9–2.8)	0.37 (0.21–0.66)	0.15 (0.01–0.35)
At 18 months of follow up	7	2.7* (1.9–2.9)	1.2 (0.9–1.8)	0.31 (0.24–0.47)	0.14 (0.07–0.26)
"Cured" patients	11	3.0 (2.1–5.8)	1.5 (0.8–2.1)	0.36 (0.17–0.63)	0.12 (0.02–0.35)
Controls	17	3.0 (2.3–5.3)	1.97† (0.8–2.6)	0.49 (0.23–1.08)	0.15 (0.04–0.29)

* Significantly higher in patients before treatment ($p < 0.05$) than after 18 months of follow up.

† Significantly decreased in patients before treatment ($p < 0.01$) compared with controls.

The Mann-Whitney U-test was used to compare differences between groups. Values are given as median (range).

of TNF α , may be a further sign of monocyte activation.

Increased monocyte activity in cancer could reflect a genetically determined increase in the activity of the monocyte population and render this group of patients more prone to developing cancer. This is because oxygen free radicals are potentially mutagenic and carcinogenic.¹² However, a more likely explanation is that the increased activity of the monocytes is a consequence of cancer cell growth and is part of the defence against cancer. Two findings in our present report strongly support the latter interpretation. One is the normal production of chemiluminescence in "cured" patients and the other is the normalisation of the chemiluminescence production in successfully treated patients. Moreover, in previous studies we observed that the increased activity was independent of cancer spread.¹¹ Indeed, increased monocyte activity in patients with cancer resulting in greater cytostatic and cytotoxic activity against established cultured tumour cells^{25–28} and increased production of TNF α ^{27,29} has been reported previously. Raised serum concentrations of TNF α in patients with solid tumours have also been reported.^{20,30}

An intriguing finding in this study was the long delay in the normalisation of the signs of monocyte activity after eradication of the tumour. Over a year elapsed before whole blood chemiluminescence returned to normal and a little under a year for TNF α . This means that in spite of successful tumour eradication and although these two variables were raised as a consequence of the presence of the tumour, the monocytes remained activated. This residual activation is probably a result of the immune memory mechanism, in which the interactions between cytokines released by the tumour cells, T lymphocyte activated macrophages, eosinophils, and neutrophils are thought to form an important effector mechanism.³¹ Although, in the present study, normalisation of chemiluminescence did not reach significance until 13 to 18 months after tumour eradication, in some patients values had returned to normal within a few months, whereas in others chemiluminescence production remained high for up to two years. To what extent the latter may be beneficial to the patient in protecting him/her against re-establishment of the cancer is uncertain. One could argue that the reason for the continuous activation of the monocytes was the fact that some cancer cells had survived although there was no clinical evi-

dence of this. However, the fact that the two tumour markers, AFP and β -HCG, were quickly normalised after orchiectomy suggests that this is not the case.

To our knowledge this is the first study showing continuous activation of the cellular mechanisms involved in the defence against cancer after eradication of the target cells. Braun *et al*³² found that monocyte activity normalised shortly after tumour eradication. In the present study, unlike that of Braun *et al*, manipulation of the cells was kept to a minimum prior to the measurement of their activity, which may have contributed to the different results obtained. We believe that the findings not only of increased production of chemiluminescence, but also of raised serum TNF α concentrations long after tumour eradication strongly support our observations, although the latter may be produced by many other cells. If our findings are confirmed in future studies they could be of great importance in unravelling the mechanisms involved in cancer defence reactions.

How are the monocytes activated in cancer and what mechanisms are involved in the continuous activation of these cells after tumour eradication? Activated monocytes can discriminate between normal and neoplastic cells and selectively bind to tumour cells.^{25,33,34} This initiates the production of reactive oxygen metabolites,^{1,34} the release of enzymes and the production of cytokines,^{13,19} some of which activate monocytes, such as interleukin-1, TNF α and colony stimulating factors.^{4,35} It seems that some patients with cancer produce TNF α as part of the host response to the presence of tumour cells, but others produce it as part of tumorigenesis.^{20,29} TNF α plays a major role in the immunoregulatory network^{17,36} and, together with interferon γ (IFN γ), induces monocytes to produce reactive oxygen intermediates.^{28,36,37} IFN γ is produced by Th 1 T helper cells and natural killer cells, among others. The continued production of these cytokines after tumour eradication might be responsible for the extended activation of monocytes, as observed in the present study.

In conclusion, whole blood lucigenin enhanced chemiluminescence and TNF α serum concentrations are raised in patients with cancer. Normalisation of these variables occurs slowly with a delay of up to a year after eradication of the tumour. We hypothesise that this reflects the continued activation of the blood monocytes and that this activation is a consequence of the continuous production of vari-

ous monocyte activating cytokines produced by cells involved in host defence against cancer spread. The capacity of an individual to mobilise these cells and to activate monocytes may determine that person's capacity to protect him/herself against cancer spread. The measurement of whole blood lucigenin enhanced chemiluminescence may be a convenient way to reflect these activities and therefore may be useful for monitoring patients with cancer.

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