Correspondence

Modification of simple and specific test for measuring lipid peroxides in plasma

In our paper, we used and recommended an iodometric (spectrophotometric) test to measure lipid peroxides in human plasma samples.[1] The use of a commercially available colour reagent (CHO-iodide; Merck, Germany) made the technique very simple and easy to use.

Not long after the paper had been published, we and others have had difficulties performing the test as it was described. Basically, the saline treated (control) sample resulted in higher absorbance than the colour reagent treated (test) sample. This was due to some as yet unidentified changes in the CHO-iodide reagent. The colour reagent presently available somehow quenches the original absorbance of plasma due to yellowish carotenoids. All our efforts to identify and eliminate this interference or change the protocol while measuring the formed I_3^- at 360 nm have failed.

We then adopted a technique, which measures iodine in the presence of starch as a blue colour iodine-starch complex at 560 nm,[2] where the natural yellowish colour of plasma does not inferfere with the measurement. This modification has been in use for six months with different batches of CHO-iodiode and produced reliable estimate of the overall oxidative capacity of plasma.

Reagents:

CHOD-iodide (Merck, Germany).

Butylated hydroxy toluene (BHT): 0·572 mg/ml in ethanol.

Starch (potato, soluble from Sigma, No. S-2630): A 1% solution is made freshly in 6·7 mM sodium chloride (39·2 mg/100 ml) by immersing into boiling water for a few minutes. After centrifugation (3 minutes at 12 000 rpm, Eppendorf centrifuge), the clear supernatant fluid is used the same day.

Procedure: Blood is taken into EDTA (1·5 mg/ml), centrifuged, and the plasma samples kept at 4°C for no longer than two days. In a small phial, place 200 μl plasma, 1·0 ml colour reagent (CHOD), 10 μl BHT and 200 μl starch solution. Mix and transfer into a disposable cuvette and read the absorbance immediately at 560 nm against distilled water. Place the cuvette immediately into dark and then read the absorbance again at 60 minutes. The difference in the absorbance represents the peroxide content of plasma. Direct calculation by using molar absorptivity is difficult due to the variation of the absorption with the source of starch. However, for one batch, the system can be calibrated by hydrogen peroxide, according to the stoichiometry of the reaction:

$$(1)\ H_2O_2 + 2I^- + 2H^+ \rightarrow I_2 + H_2O$$

$$(2)\ I_2 + I^- \rightleftharpoons I_3^-$$

Absorption changes are linear between the amounts of peroxide added (0·2–1 nmol) and the I_3^- produced.

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Parathyroid hormone related protein (PTHrP) in hypercalcaemia of lymphoproliferative disease

Bolo-Deoku *et al* have shown that PTHrP was not implicated in a case of hypercalcaemia in Hodgkin's disease, thus expanding the very limited literature on PTHrP in lymphoproliferative disease.[1] We report a case of hypercalcaemia in chronic lymphocytic leukaemia (CLL) where the same conclusion was reached.

Eight years after presentation, a patient with stage IV c B-CLL developed acute severe low back pain due to vertebral body collapse. Generalised loss of bone density was apparent radiologically, but no discreet osteolytic lesions were found. Corrected serum calcium concentration was 2·94 mmol/l (range 2·26–2·60), phosphate activity 1·31 mmol/l (range 0·8–1·4), alkaline phosphatase 84 IU/l (range 30–115), and renal function was normal. Vigorous intravenous hydration and intensified treatment with corticosteroids failed to control the hypercalcaemia and a peak corrected serum calcium of 3·29 mmol/l was reached before effective treatment with pamidronate sodium was started. Prolymphocytoid transformation and myeloma were excluded and there was no evidence of coincidental malignancy. Serum 25-hydroxycholecalciferol was 11·8 ng/ml (range 8–50), PTH 1·7 pmol/l (range 0·9–5·4), and PTH-related protein (PTHrP) was less than 0·23 pmol/l (normal).

While lymphotoxin (tumour necrosis factor β), interleukins 1 and 6, and prostaglandins have a well defined role in the bone disease of myelomatosis,[2] the mechanisms underlying dysfunctional osteoclast activity in other lymphoproliferative disorders[3] are unknown. Although larger studies must be performed, it seems unlikely that PTHrP has an important role.

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Value of PCNA and Ki67 staining in breast cancer

The failure of PCNA to reflect proliferative activity, as measured by Ki67 or mitotic counting in breast tumours, documented by Leonardi and colleagues,[1] is disappointing. We have found a weak positive correlation between PCNA staining and Ki67 staining in both benign and malignant breast tumours (personal observation).

To some degree such observations may be related to the techniques used. Our practice with both Ki67 and PCNA is to count areas of highest antigen expression after scanning the slide at low magnification. This gives a measure of the maximum proliferative activity in any given tumour and obviates the need to decide how many areas of low and high concentration to include (not to mention the intermediate areas).

Using this method, we found no correlation between PCNA and mitotic count in carcinomas but a weak correlation ($r = 0{\cdot}43$, $p < 0{\cdot}005$) was obtained between Ki67 and PCNA in benign and malignant tumours.

As well as its extreme sensitivity to prolonged fixation and to heating on the slide before staining,[2] another notable factor with PCNA we have observed is that prior freezing of tissue abolishes PCNA immunodetection, precluding the use of frozen tissue subsequently processed to paraffin wax such as obtains with biopsy specimens and small tumours.

These factors tend to reduce the efficacy of PCNA immunostaining, but the main problem with PCNA appears to be its overexpression. It is apparent when assessing PCNA expression that mitotic figures are frequently unstained. This is consistent with its described expression in the S-phase under optimal conditions.[3] Yet PCNA expression is frequently higher than Ki67 index[1 2] as we have found, and higher than thymidine labelling in benign breast tissue.[4] As Ki67 and thymidine labelling are restricted to the cell cycle only, PCNA must therefore persist in non-cycling cells but in a variable manner, losing its association with mitotic activity and making its correlation with Ki67 expression tenuous.

This suggests that although PCNA is to some extent comparable with Ki67 it is influenced by factors as yet undefined to such an extent that it loses its usefulness in quantifying proliferative activity.

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Dr Barbareschi comments:

Dr Sullivan's letter, which suggests a weak correlation between PCNA and Ki67 immunostaining in benign and malignant breast tumours, agrees with our paper where we concluded that in breast cancer PCNA immunostaining is not a substitute for Ki67

labelling.[1] PCNA immunohistochemical expression (evaluated with the PC10 monoclonal antibody) seems to be related to cellular proliferation in many normal tissues and in some neoplasms,[2] such as gastrointestinal lymphomas,[3] central nervous system tumours,[4] lung neuroendocrine neoplasms,[5] and prostatic carcinomas.[6] However, in other tumours, like breast and gastric cancer, PCNA (PC10) expression seems aberrant and not strictly related to proliferative activity.[1 7 8]

Various factors unrelated to cell proliferation may influence the immunohistochemical expression of PCNA, including post-transcriptional regulation (and deregulation) of the PCNA gene,[8 9] long half-life of the PCNA protein,[10] involvement of PCNA protein in DNA repair synthesis,[11] and tissue and section processing—type and ionic strength of the fixatives, fixation time, section heating, immunohistochemical techniques.[8 12 13]

Further problems in PCNA immunohistochemical staining, as in other kinetic quantitative immunohistochemical studies, concern evaluation and scoring methods.[14 15] Should we use quantitative or semiquantitative methods? How many cells should be counted? Which tumour areas should be evaluated (the most positive or random selected areas)? Which immunoreactive cells should be evaluated (all positive cells or only the most intensely stained)?

Particular attention should be also drawn to the kind of antibody used to localise PCNA. Different staining patterns may be seen with different antibodies, and this may add to conflicting and confusing results.[14]

In our opinion PCNA immunostaining should be evaluated with great caution and in some fields even with scepticism. More work is needed to assess the extent and range of PCNA staining in different tissues and lesions (neoplastic and non-neoplastic). PCNA counts should be evaluated concurrently with the different anti-PCNA available antibodies and the results should be compared with other "proliferation markers" and especially with clinical data. The possibility that PCNA immunostaining may have diagnostic[5] or prognostic value[7] is intriguing and carefully performed clinicopathological studies are needed to assess this possibility further. This will be the only way to know if we are faced with an interesting but clinically worthless tool or with an important test to be added to the routine evaluation of neoplasms.

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AgNOR quantification in tumour pathology: What is actually evaluated?

The interest of pathologists in interphase silver stained nucleolar organiser regions (AgNORs) has intensified since it was shown that malignant cells frequently have higher AgNOR numbers compared with corresponding benign or normal cells. Moreover, interphase AgNOR numbers are closely related to cell proliferative activity, suggesting that this parameter might also have prognostic importance.

Nucleolar organiser regions (NORs) are chromosomal segments which contain ribosomal genes. NORs are associated with a group of argyrophilic proteins, and can be visualised by silver staining in routinely processed cytological and histological samples. At light microscopic level AgNORs appear as well defined black dots, which in interphase cells are exclusively distributed throughout the lighter stained nucleoli. Each black dot corresponds, at the ultrastructural level, to a fibrillar centre with the surrounding dense fibrillar component. The number of AgNORs in quiescent cells is generally low (most lymphocytes or stromal cells have only one), while in proliferating cells, such as cancer cells, a high AgNOR number is present.

Over the past six years the silver staining technique has become widespread among pathologists, but the lack of a standardised staining protocol has led to misinterpretation of structures evaluated by different authors.[1] Looking in fact at the micrographs reported—for example, by Giri *et al* (breast carcinoma)[2] Öfner *et al*, (colonic carcinoma)[3] Cheville *et al*, (prostatic carcinoma)[4] and Kaneko *et al* (lung carcinoma),[5] it is evident that not just the AgNORs, but the whole nucleoli have been stained by silver and counted as NORs.

The selective visualisation of AgNORs is subject, apart from the fixative used, to the temperature and temporal length of the staining reaction. These two variables are inversely related to each other: the higher the temperature, the shorter the time required for NOR silver staining. When the staining reaction is prolonged beyond the time for selective visualisation of NORs, all the other nucleolar structures are progressively stained, until the whole nucleolus appears homogeneously stained by silver. It is therefore evident that different nucleolar structures have been stained and counted in various laboratories, and this has caused disagreement about AgNOR numbers reported in individual studies on the same neoplastic lesions.

In a recent investigation it was shown that the total interphase AgNOR area was closely related to the whole nucleolar area stained by silver when staining was prolonged beyond the optimal time for selective interphase NOR visualisation.[6]

To obtain comparable data between different laboratories the whole nucleolus ought to be silver stained and the area occupied by the silver stained nucleoli per cell measured using image analysis instead of AgNOR counting. Because AgNOR area and nucleolar area are so strictly related to each other, the morphometric analysis of silver stained nucleoli will certainly have the same clinical and biological relevance demonstrated for interphase AgNORs.

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Method for grading breast cancer

Parham and colleagues[1] have proposed a new and "simplified" method for grading breast cancer and claim that it is superior to the Bloom and Richardson method,[2] which they rightly criticise for its lack of precision. We agree entirely with this criticism, but are rather surprised that they do not refer to our recent publication in which, for precisely this reason, we have devised modifications which provide objective criteria for the evaluation of the three morphological components of histological grade.[3] We have shown in a study of over 1500 patients that histological grade, using this method, provides powerful prognostic information, and in combination with tumour size and lymph node stage, forms the Nottingham Prognostic Index which can be used by clinicians to stratify patients for