breast cancer cell proliferation *in vitro*: a possible model for tamoxifen tumor flare. *Eur J Cancer Clin Oncol* 1984, **20**, 1419–1424.
27. McIntosh IH, Thynne GS. Tumor stimulation by antiestrogens. *Br J Surg* 1977, **64**, 900–901.
28. Plotkin D, Lechner JJ, Jung WE, Rosen PJ. Tamoxifen flare in advanced breast cancer. *JAMA* 1978, **240**, 2644–2646.
29. Powles TJ, Tillyer CR, Jones AL, *et al.* Prevention of breast cancer with tamoxifen—an update on the Royal Marsden Hospital pilot programme. *Eur J Cancer* 1990, **26**, 680–684.

Acknowledgements—We thank Jane Wegenke and JoAnne Barrix for collecting and storing serum samples. We also thank the family of Eileen Henrich for their gift to the UWCCC to provide equipment for the measurement of tamoxifen.

Eur J Cancer, Vol. 26, No. 8, pp. 888–891, 1990.
Printed in Great Britain

0277-5379/90 $3.00 + 0.00
Pergamon Press plc

Cellular Retinol Binding Protein and Breast Carcinoma

Rajeshwari R. Mehta and Gloria D. Hart

Cellular retinol binding protein (C-RBP) levels were measured in 87 malignant and 18 non-malignant breast cancer tissues. C-RBP, sedimenting in the '2S' region on 5–20% sucrose density gradients, was detectable in 70% of malignant tissues examined. None of the non-malignant tissues contained detectable C-RBP. No significant association between tumour steroid receptors status, patients' obesity or menopausal status and C-RBP contents was observed. However, patients with stage IV disease had higher C-RBP levels than patients at stages II and III ($P < 0.0001$), which suggested altered intracellular mobilization of retinol in the tumour, probably as an indirect consequence of inadequate nutrient intake.
Eur J Cancer, Vol. 26, No. 8, pp. 888–891, 1990.

INTRODUCTION

VITAMIN A and retinoids are important in the growth and differentiation of epithelial cells [1]. Under experimental conditions, cells deprived of vitamin A are prone to malignant transformation [2, 3]. Dietary vitamin A deficiency increases the frequency of spontaneous and virus or carcinogen induced epithelial malignant tumours in laboratory animals [4–8]. A synthetic analogue of vitamin A (4-hydroxy-phenylretinamide [4HPR]) is effective against carcinogen N-nitromethylsourea (NMU) induced tumours in rats [9, 10]. Also, synergistic or additive inhibitory action of 4HPR and ovariectomy or anti-oestrogen has been reported [10]. However, reports on the chemopreventive action of vitamin A in humans are conflicting. Prospective and retrospective studies in humans suggest a relation between vitamin A deficiency and malignancies of epithelial origin [2, 11]. Epidemiological studies on a specific geographic population also suggested an inverse association between blood levels of vitamin A and risk of developing cancer [12–16]. In contrast, Willett *et al.* [17] failed to observe such a relation between risk of developing cancer and blood vitamin A levels. Prospective studies on blood levels of vitamin A, β-carotene or retinol binding protein (RBP) failed to correlate vitamin A levels and development of cancer [17].

We have studied plasma RBP in premenopausal breast carcinoma patients receiving adjuvant chemotherapy. Women with lower plasma RBP during the course of chemotherapy had tumour recurrence earlier than those who had higher RBP levels [18]. No such correlation between plasma vitamin A or β-carotene levels and time to tumour recurrence was observed. In the present study, we measured cellular RBP (C-RBP) levels in malignant and non-malignant breast tissues. We investigated correlations between C-RBP status in the tumour and the patient's menopausal, steroid receptor and disease status.

MATERIALS AND METHODS

Clinical materials

Clinical material was available through the cooperation of surgeons and pathologists associated with the Division of Surgical Oncology, University of Illinois Hospital, and Cook County Hospital. Malignant ($n = 87$) and non-malignant ($n = 18$) breast tissues were obtained from women with confirmed diagnosis of cancer undergoing biopsy or mastectomy. Following surgical excision, tissues were immediately transported on ice to our laboratory, freed from connective tissues and blood vessels, and stored at −80°C until assayed for receptors or C-RBP. Information about the patients' age, body weight, height, menopausal status and clinical staging of disease were maintained in the divisional computer system.

Chemicals

[^{3}H]-oestradiol (2,4,6,7,^{3}H-N-oestradiol, specific activity 362 × 10^{10} Bq/mmol), [^{3}H]progesterone (17-N-methyl-^{3}H, 322 × 10^{10} Bq/mmol) and ^{3}H-retinol (74 × 10^{10} Bq/mmol) were obtained from New England Nuclear. All assays were performed with 'Tris'–EDTA buffer (10 mmol/l Tris–HCl, 1.5 mmol/l

Correspondence to R.R. Mehta, Department of Surgery (M/C 820), University of Illinois at Chicago, 840 South Wood Street, Chicago, IL 60612, U.S.A.
R.R. Mehta is at the Specialized Center for Cancer Research and Education, the Division of Surgical Oncology of the University of Illinois Medical Center, G.D. Hart is at Cook County Hospital, and the authors are also associated with the West Side Veterans Administration, The Hektoen Institute of Medical Research, Chicago, Illinois, U.S.A.

Table 1. Steroid receptors and C-RBP in human breast tumour tissue

Steroid receptor status	No. RBP⁺ total number	C-RBP levels (pmol/mg protein)
ER⁺	35/43 (81%)	1.83 (0.31, 35)†
ER⁻	26/44 (59%)	1.94 (0.39, 26)
PR⁺	26/33 (79%)	1.62 (0.34, 26)
PR⁻	27/44 (61%)	2.00 (0.38, 27)
ER⁺PR⁺	24/28 (86%)*	1.70 (0.36, 24)
ER⁺PR⁻	8/12 (67%)	2.33 (0.74, 8)
ER⁻PR⁻	19/32 (59%)	1.89 (0.47, 19)
ER⁻PR⁺	2/5 (40%)*	0.62 (0.63, 2)

*$P < 0.001$.
†Mean (S.E., n).

EDTA, pH 7.4, containing monothioglycerol 10 mmol/l, 10% glycerol and 10 mmol/l sodium molybdate).

Steroid receptor analysis

Steroid receptors were analysed by the multipoint titration method [19]. 0.5–2.0 g biopsy or mastectomy specimens were immediately placed in a sterile container and transported to our laboratory on dry ice. Tumours were freed from fat and other connective tissues and stored at −80°C then weighed, pulverized into powder on liquid nitrogen and homogenized on ice in a 'Polytron PT-10' homogenizer with three 10 s bursts and a 30 s cooling period between each burst. The homogenate was centrifuged at 105 000 *g* for 60 min at 4°C. Any lipid layer observed on the surface was removed with a spatula.

For steroid receptor analysis, aliquots of supernatant cytosol were incubated with increasing concentrations (0.1–5 nmol/l) of appropriate radiolabelled ligand alone or in the presence of 100-fold excess unlabelled ligand at 4°C for 16 h. To separate bound from free ligand, 'Dextran'-coated charcoal (DCC; 0.025% Dextran 0.25% 'Norit A' in assay buffer) was used. The radioactivity in the supernatant fluid was counted and the specific binding of ligand was subjected to Scatchard analysis.

Cellular retinol binding protein

RBP in the cytosol was analysed on sucrose density gradients. An aliquot of cytosol was incubated at 4°C for 4 h with 60 pmol ^{3}H-retinol alone or in the presence of 100-fold excess unlabelled retinol. Reaction mixture was treated with DCC for 10 min. Following centrifugation, aliquots of supernatant were layered on a 5–20% sucrose gradient and centrifuged at 149 000 *g* for 16 h. The gradients were fractionated and the radioactivity in each fraction was counted. The binding protein was detected as a distinct radioactive peak, replaced by the 100-fold excess unlabelled retinol. The radioactivity in the area under the peak was used to quantify the binding, which is expressed as pmol of ligand bound per mg cytosol protein. Protein contents were determined by the method of Lowry *et al.*

Tissues containing more than 10 fmol/mg protein steroid receptors (oestrogen [ER] and progesterone [PR]) were considered positive (ER⁺, PR⁺). For RBP, tissues showing any detectable ^{3}H-retinol peak (in the '2S' region) replaced by unlabelled retinol were considered C-RBP⁺.

Statistical significance was analysed by chi-squared or *t* tests.

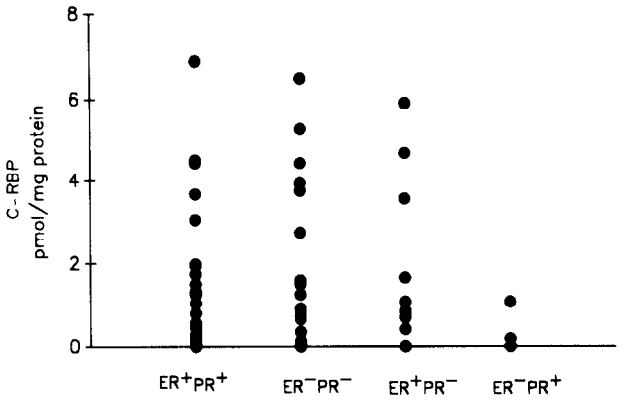

Fig. 2. Frequency distribution of C-RBP⁺ tumours in relation to steroid receptor status.

RESULTS

Steroid receptors and C-RBP

Overall, of 87 malignant tumours, 49% (43/87) were ER⁺ and 43% (33/77) were PR⁺. RBP distinctly sedimenting in the 2S region on a 5–20% sucrose density gradient (Fig. 1) was detectable in 70% (61/87) of malignant tumours. The mean contents were 1.77 [S.E. 0.21] pmol/mg protein, ranging between 0.03 to 6.8. None of the non-malignant breast tissues contained detectable levels of steroid receptors or C-RBP.

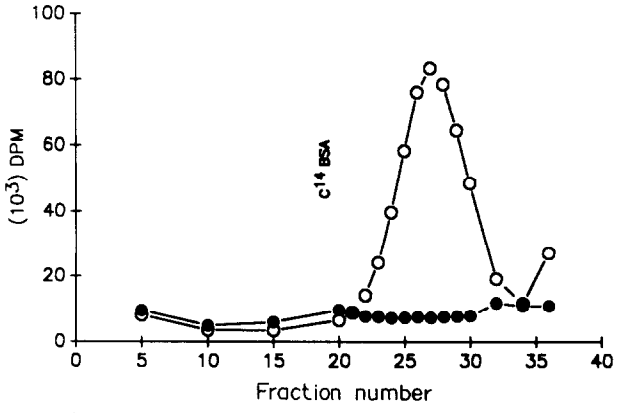

Fig. 1. C-RBP profile on 5–20% sucrose density gradient. Cytosol from malignant tumour was incubated with ^{3}H-retinol alone (○) or in the presence of unlabelled retinol (●) for 4 h at 4°C. DPM = disintegrations per min, BSA = bovine serum albumin.

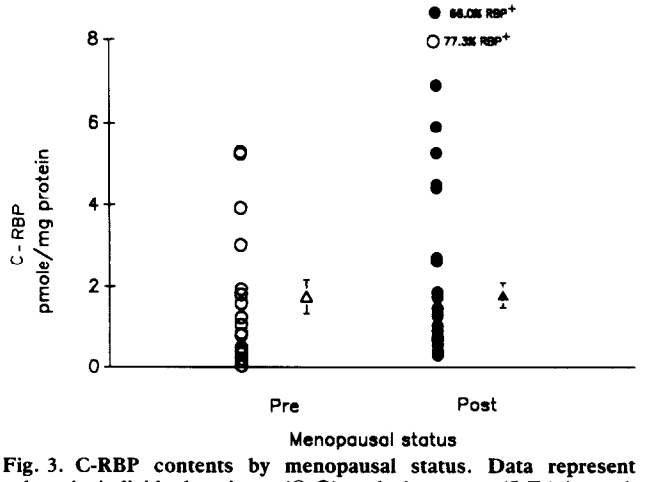

Fig. 3. C-RBP contents by menopausal status. Data represent values in individual patients (○,●) and also mean (S.E.) in each group (△, ▲).

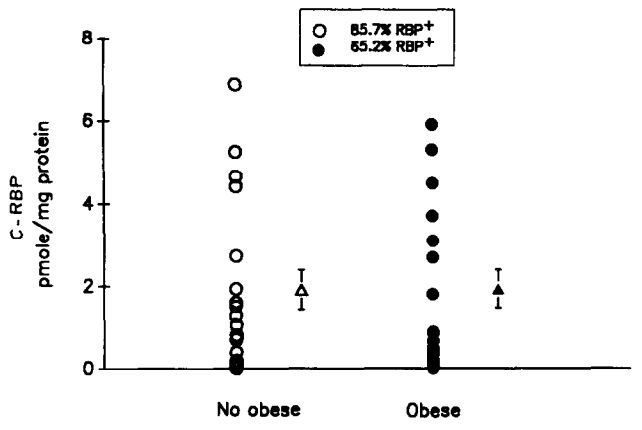

Fig. 4. C-RBP in tumours of obese and non-obese women.

The frequency of RBP$^+$ tumours in relation to their steroid receptor status is shown in Table 1. Overall, 81% of ER$^+$ and 59% of ER$^-$ tumours and 79% of PR$^+$ and 61% of PR$^-$ tumours were also C-RBP$^+$. The frequency distribution of C-RBP$^+$ tumours in relation to their ER and PR status is shown in Fig. 2. No significant difference in the distribution of C-RBP$^+$ tumours was observed in tumours with different steroid receptor status. The frequency of C-RBP$^+$ tumours was higher in ER$^+$ PR$^+$ than in ER$^-$ PR$^+$ tumours (Table 1).

C-RBP and menopausal status

Patients' menopausal status was determined from the history of occurrence of last known menstrual period for 72 women. 22 were premenopausal and the remaining 50 were postmenopausal (Fig. 3). 17 (77%) premenopausal women and 34 (68%) post-menopausal women had detectable C-RBP levels in their tumour specimen. The frequency of C-RBP$^+$ tumours and the mean concentration of C-RBP were not significantly different in premenopausal compared with post-menopausal women.

C-RBP and obesity

Patients were considered obese if they weighed over 120% of ideal body weight (IBW) according to the height and weight table from Metropolitan Life Insurance. Body weight and height were known in 45 women: 24 were obese (Fig. 4). The frequency of C-RBP$^+$ tumours and mean C-RBP levels were not significantly different in obese and non-obese women.

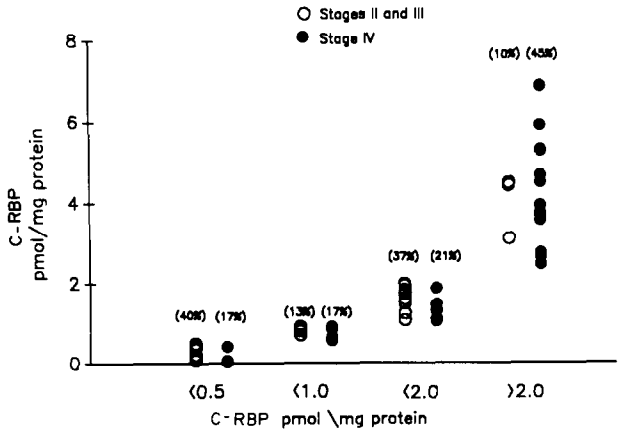

Fig. 5. Frequency distribution of C-RBP$^+$ tumours in relation to disease status.

C-RBP and disease status

Clinical staging (TNM classification) was determined by the surgical oncologists in 75 patients. 43 had stage II and III disease, the remaining 32 were in stage IV with distant metastasis. 30 (70%) of the stage II–III women and 29 (91%) of the stage IV women were positive for C-RBP (Fig. 5). The frequency of tumours containing more than 2.0 pmol/mg protein was higher (10% vs. 45%, $P < 0.0001$) in stage IV patients than in patients at stages II–III. Also, patients at stage II–III disease more frequently (40% vs. 17%) had C-RBP contents under 0.5 pmol/mg protein than those patients with metastatic disease. The mean content of C-RBP was significantly higher ($P < 0.01$) in patients at stage IV (2.35 [S.E. 0.37] pmol/mg protein) disease than in patients at stage II–III disease (1.18 [S.E. 0.21] pmol/mg protein).

DISCUSSION

In vivo, retinoids or vitamin A mediate their actions via three different specific proteins: plasma RBP, which transports retinoids to the target sites, C-RBP and cellular retinoic acid binding protein, which are probably responsible for intracellular mobilization of retinoids at genomic sites, actual interaction and genomic regulation of vitamin A function [20]. In malignant breast tumours, both RBP and retinoic acid binding proteins have been reported [21, 22]. However, no attempt has been made to establish the significance of C-RBP in the prognosis of this disease. On the other hand, increased levels of cellular retinoic acid binding protein are associated with histopathologically well-differentiated tumours [22].

We aimed to assess the physiological importance of C-RBP in the clinical representation of breast carcinoma. RBP sedimenting in the '2S' region on 5–20% sucrose density gradients was detected in 70% of malignant tumours studied, and none of the non-malignant tissues contained C-RBP levels. No significant association between presence/absence of C-RBP levels or actual C-RBP contents and other prognostic factors such as tumour receptor status or patients' obesity or menopausal status was found. However, C-RBP levels in relation to patients' clinical disease status were interesting. Patients at stage IV with distant visceral metastasis of breast carcinoma had significantly higher C-RBP levels than patients with no clinical evidence of metastatic disease (stages II–III). In experimental animals, C-RBP levels increase when animals are fed diets deficient in vitamin A [12]. Thus, in our study, increased C-RBP levels in stage IV patients may reflect inadequate vitamin A delivery at the cellular level. Previous studies [18] on plasma RBP in premenopausal stage II–III breast cancer patients receiving chemotherapy showed that patients with lower RBP had early tumour recurrence compared with those with higher RBP levels. Further studies on plasma prealbumin vitamin A and total protein levels suggested that a low level of plasma RBP may be a result of low dietary intake of nutrients in patients with advanced disease, causing mild protein malnutrition. Data from our previous study and the current studies indicate that, in breast cancer patients with advanced disease, mild protein malnutrition due to inadequate dietary intake may result in lower plasma RBP levels, which in turn results in vitamin A deficiency at cellular levels and increased C-RBP sites. Thus, altered vitamin A availability at the genomic site may influence the normal differentiation of breast cells.

1. Moore T. Effects of vitamin A deficiency in animals. In: Sebrell WH, Harris RS, eds. *The Vitamins*, Vol. 1. New York, Academic Press, 1967, 245–266.
2. Bjelke E. Dietary vitamin A and human lung cancer. *Int J Cancer* 1975, **15**, 561–567.
3. Sporn MB, Dunlop NM, Newton DL, Smith JM. Prevention of chemical carcinogens by vitamin A and its synthetic analogs (retinoids). *Fed Proc Fed Am Soc Exp Biol* 1976, **35**, 1332–1338.
4. Fujimaki Y. Formation of gastric carcinoma in albino rats fed on deficient diets. *J Cancer Res* 1926, **10**, 469–477.
5. Heilman C, Swarm RL. Effect of thirteen cis vitamin A acid on chondrosarcoma. *Fed Proc Fed Am Soc Exp Biol* 1975, **34**, 882.
6. McMichael H. Inhibition of growth of shope rabbit papilloma by hypervitaminosis A. *Cancer Res* 1965, **25**, 947–955.
7. Moon RC, Grubbs CJ, Sporn MB. Inhibition of 7,12-dimethylbenz(a)anthracene induced mammary carcinogenesis by retinyl acetate. *Cancer Res* 1976, **36**, 2626–2630.
8. Moon RC, Grubbs CJ, Sporn MB, Goodman KY. Retinyl acetate inhibits mammary carcinogenesis induced by N-methyl-N-nitrosourea. *Nature* 1977, **267**, 620–621.
9. Moon RC, Thompson HJ, Becci PJ, *et al.* N-(H-hydroxyphenyl) retinamide, a new retinoid for prevention of breast cancer in rats. *Cancer Res* 1979, **39**, 1339–1346.
10. Mehta RG, Moon RC. Inhibition of DNA synthesis by retinyl acetate during chemically induced mammary carcinogenesis by combination N-(4-hydroxylphenyl) retinamide and ovariectomy. *Cancer Res* 1980, **40**, 1109–1111.
11. Romney SL, Palan PR, Duttagupta C, *et al.* Retinoid and prevention of cervical dysplasias. *Am J Obstet Gynec* 1981, **141**, 890–894.
12. Wald N, Idle M, Boreham J, Bailey A. Low serum vitamin A and subsequent risk of cancer: preliminary results of prospective study. *Lancet* ii, 813–815.
13. Bernstein A, Harris B. The relationship of dietary and serum vitamin A to the occurrence of crucial intraepithelial neoplasia in sexually active women. *Am J Obstet Gynec* 1984, **148**, 309.
14. Kark JD, Smith AH, Switzer BR, Hames CG. Serum vitamin A (retinol) and cancer incidence in Evans County, Georgia. *J Natl Cancer Inst* 1981, **66**, 7–16.
15. Peto R, Doll R, Buckley JD, Sporn MB. Can dietary beta carotene naturally reduce human cancer rates? *Nature* 1981, **290**, 201–208.
16. Willett WC, Polk BF, Underwood BA. Relationship of serum vitamin A and carotenoids to the risk of cancer. *New Engl J Med* 1984, **310**, 430–434.
17. Willett WC, Polk BF, Underwood BA, Hames CG. Hypertension detection and follow-up program study of serum retinol, retinol binding protein, total carotenoids and cancer risk: a summary. *J Natl Cancer Inst* 1984, **73**, 1459–1462.
18. Mehta RR, Hart G, Beattie CW, Das Gupta TK. Significance of plasma retinol binding protein levels in recurrence of breast tumors in women. *Oncology* 1987, **44**, 350–355.
19. Mehta RR, Das Gupta TK. Antiestrogen binding sites in microsomial fractions of malignant and non-malignant human breast tissues. *Breast Cancer Res Treat* 1987, **9**, 61–67.
20. Ong D. Vitamin A binding proteins. *Nutrition Rev* 1985, **43**.
21. Mehta RG, Kute TE, Hopkins M, Moon RC. Retinoic acid binding proteins and steroid receptor levels in human breast cancer. *Eur J Cancer Clin Oncol* 1982, **18**, 221–226.
22. Skoog L, Humla S, Klintenberg C, Pasqual M, Wallgren A. Receptors for retinoic acid and retinol in human mammary carcinomas. *Eur J Cancer Clin Oncol* 1985, **21**, 901–906.

Acknowledgement—This study was in part supported by R29 CA 46423 and PO1 CA 31827, the Seymour Engel Memorial Fund for Cancer Research and the Ladies Auxillary of the VFW.

Eur J Cancer, Vol. 26, No. 8, pp. 891–895, 1990.
Printed in Great Britain

0277–5379/90 $3.00 + 0.00
Pergamon Press plc

Polyethylene Glycol-L-asparaginase versus Native L-asparaginase in Canine Non-Hodgkin's Lymphoma

Erik Teske, Gerard R. Rutteman, Peter van Heerde and Wim Misdorp

42 dogs with non-Hodgkin's lymphoma (NHL) were randomized for treatment with either PEG-L-asparaginase 10 IU/kg intramuscularly (n = 22) or L-asparaginase 400 IU/kg intraperitoneally (n = 20). Another 20 dogs were treated with either PEG-L-asparaginase 30 IU/kg (n = 10) or L-asparaginase 400 IU/kg (n = 10). Each treatment protocol consisted of two asparaginase treatments followed by a 10-week period of induction chemotherapy and then maintenance on asparaginase until progression occurred. No significant differences were found between treatments in the response rates after 2 weeks of asparaginase therapy or in the time to relapse, the time to treatment failure or the remission period. The reaction to asparaginase after the initial 2 weeks was a prognostic factor for the total duration of remission under asparaginase maintenance therapy. No side-effects were noted in the dogs treated with PEG-L-asparaginase, whereas 14 (48%) of the L-asparaginase treated dogs had side-effects related to this drug, including anaphylactic shock (9), anorexia or vomiting (4), hypersensitivity-related oedema (3), seizures (1) and acute pancreatitis (1). No abnormalities in clotting times, fibrinogen levels or antithrombin-III levels were found in any of the 62 dogs. PEG-L-asparaginase has the same anti-tumour activity as native L-asparaginase in dogs with NHL, but lacks side-effects.
Eur J Cancer, Vol. 26, No. 8, pp. 891–895, 1990.