ACUTE PHASE REACTANTS CERULOPLASMIN AND HAPTOGLOBIN AND THEIR RELATIONSHIP TO PLASMA PROSTAGLANDINS IN RABBITS BEARING THE VX_2 CARCINOMA*

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Results of a series of studies on the cause of the hypercalcemia that occurs in mice bearing the $HSDM_1$ fibrosarcoma (1-6) and in rabbits carrying the VX_2 carcinoma (7-9) have led us to conclude that these two tumors synthesize and secrete large amounts of prostaglandin E_2 (PGE_2) into plasma.[1] PGE_2 is a potent bone resorption-stimulating agent in vitro (2, 10), and this prostaglandin and its metabolites are found in elevated concentrations in the plasma of tumor-bearing animals (2, 3, 6-8, 11). Because of the rapid clearance and metabolism of PGE_2, measurements in plasma of the metabolite, 13,14-dihydro-15-keto-PGE_2(PGE_2-M), give a more accurate estimate of PGE_2 secretion than do measurements of the primary prostaglandin itself (6, 8, 11, 12). Studies on the time-course of the development of elevated plasma calcium concentrations and hyper-prostaglandinemia, as well as investigations using two inhibitors of prostaglandin synthesis, indomethacin (1-3, 7) and hydrocortisone (6, 8, 13), support the hypothesis that the hypercalcemic syndrome in these tumor-bearing animals is due to the secretion of PGE_2 by the tumor. A similar pathophysiologic mechanism may explain in part the hypercalcemia that occurs in certain patients with cancer (14-16).

The present investigation was initiated because of the observation that the plasma from rabbits bearing the VX_2 carcinoma became faintly blue about 1 wk after tumor implantation, and this color increased markedly and became intense by 3-4 wk. The time-course of the appearance and increase in the blue color in plasma was similar to that which we had previously noted for PGE_2-M. We therefore undertook to identify the blue material in plasma and to examine the relationship of its increase to prostaglandin metabolism. Our findings indicate that the material is ceruloplasmin, that its rise correlates closely with plasma concentrations of PGE_2-M, and that both PGE_2-M and ceruloplasmin increase in the plasma of tumor-bearing rabbits before the development of hypercalcemia.

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[1] *Abbreviations used in this paper:* dl, deciliter; PGE_2, prostaglandin E_2; PGE_2-M, 13,14-dihydro-15-keto-PGE_2.

Ceruloplasmin is a blue, copper-containing α_2-globulin which possesses intrinsic oxidase activity (17–19). Its concentration in plasma is known to vary in a variety of physiologic states and in disease (18). Decreased plasma levels have been detected in Wilson's disease, nephrosis, and malabsorption syndromes, and elevated concentrations are seen in acute and chronic infections, rheumatoid arthritis, pregnancy, during estrogen administration, and in patients with a variety of different tumors. The mediators which control ceruloplasmin synthesis and secretion by the liver in health and disease have not been clearly defined.

Plasma concentrations of haptoglobin, another α_2-globulin which binds hemoglobin, are known to vary in parallel with ceruloplasmin in certain states of disease, notably acute and chronic infection, and in patients with certain malignancies (20). Furthermore, it has been reported that administration of PGE_1 can elevate the concentration of haptoglobin in the serum of rabbits (21). We therefore measured haptoglobin as well as ceruloplasmin in the plasma of rabbits bearing the VX_2 carcinoma, and we found elevations which paralleled those of the copper-containing protein. A preliminary presentation of these findings has been made (22).

Materials and Methods

Animals. The VX_2 carcinoma was passed serially in female albino rabbits by methods previously described in detail (7). In experiments in which rabbits were treated with indomethacin from the time of tumor implantation, the drug was administered orally in an average daily dosage of 10–40 mg/rabbit/24 h. Indomethacin was incorporated into a known amount of pulverized Purina Lab Chow for Rabbits (Ralston Purina Co., St. Louis, Mo.) which the animals consumed essentially completely each 24 h (7). In experiments in which rabbits were treated with indomethacin intermittently after the development of hypercalcemia, the drug was suspended in 15% gelatin and administered by subcutaneous injection twice a day in a daily dosage of 10–20 mg/rabbit. The animals weighed 2.5–3.0 kg. For periods up to 4 wk after tumor implantation, these rabbits did not become azotemic as determined by measurements of plasma urea nitrogen and creatinine. The exact schedules by which indomethacin was given are indicated in Results.

Blood Collection. Blood was collected from a marginal ear vein or by cardiac puncture into heparinized tubes or syringes. Plasma was separated immediately by centrifugation at 4°C.

Ceruloplasmin. The concentrations of ceruloplasmin in plasma were estimated by two independent methods. The first method used the procedure of Sunderman and Nomoto (23) which depends on the *p*-phenylenediamine oxidase activity of ceruloplasmin. The standard was human ceruloplasmin, type III, from Sigma Chemical Co., St. Louis, Mo. (lot 114C-0237-1). The second method used Laurell's electroimmunoassay technique (24, 25). The antiserum was prepared against purified human serum ceruloplasmin in goats, and it was obtained from Atlantic Antibodies, Westbrook, Maine. This antiserum cross-reacted sufficiently with rabbit ceruloplasmin to provide easily identified rockets. The immunoassay results were expressed as a percentage of the basal ceruloplasmin concentration before tumor implantation for each animal.

Immunochemical Measurements of Plasma Haptoglobin and Albumin. The concentrations of haptoglobin and albumin in plasma were measured by electroimmunoassay (24, 25) using antisera prepared against analogous purified human serum proteins in goats (Atlantic Antibodies). As with ceruloplasmin, these antisera cross-reacted sufficiently with the corresponding rabbit plasma proteins to give clear rockets. The results were expressed as a percentage of the basal concentration of that protein before tumor implantation for each animal.

Prostaglandin Metabolite. The metabolite of PGE_2, PGE_2-M, was measured in plasma by radioimmunoassay (26). The anti-PGE_2-M cross-reacted with 13,14-dihydro-PGE_2, 15-keto-PGE_2, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-PGA_2, PGE_2, and PGA_2 0.2, 7.0, 5.0, 0.4, 0.1, and 0.08%, respectively (26). Plasma for PGE_2-M assay was extracted with 3 vol of methylal-ethanol and concentrated as described previously for human samples (27). Several extracts of

rabbit plasma were assayed with anti-13,14-dihydro-15-keto-$PGF_{2\alpha}$. The 13,14-dihydro-15-keto-PGE_2 gave a cross-reaction with this anti-$PGF_{2\alpha}$ metabolite of ≅3% (28). These simultaneous radioimmunoassays demonstrated that it was the PGE_2 metabolite, not the $PGF_{2\alpha}$ metabolite, that was being measured in the experiments in this report. Similar quantitative results for plasma PGE_2-M have been measured in rabbits basally and during the first 3 wk after VX_2 tumor implantation using high performance liquid chromatography and gas chromatography-mass spectrometry (11). The sensitivity of the radioimmunoassay method was 15 pg of PGE_2-M/ml rabbit plasma, and the precision of a measured value was ±20%.

Calcium. The concentration of calcium in plasma was measured in duplicate by automatic fluorometric titration with a Corning model 940 calcium analyzer (Corning Medical, Corning Glass Works, Medfield, Mass.).

Statistical Method. Where appropriate, when groups of rabbits were studied, the results were subjected to an analysis of variance, and the standard errors were calculated from the residual error term of that analysis.

Results

Plasma Ceruloplasmin. The concentration of ceruloplasmin in the plasma of 12 normal control rabbits was 37 ± 4 mg/deciliter (dl) (mean ± SE), as measured by its *p*-phenylenediamine oxidase activity. This value is similar to that found in normal human serum, 31.5 ± 5 mg/dl (mean ± SD), using the same technique (23).

The rise in plasma ceruloplasmin as a function of time after implantation of VX_2 tumor cells in three rabbits is shown in Fig. 1. A marked increase of 10 to 20 times the basal concentration was seen 3–4 wk after tumor implantation. There was generally good agreement between the results obtained on the same samples by both the chemical and immunological assay methods (Fig. 1). The largest discrepancy observed in over 25 rabbits studied was that seen in rabbit 224 (Fig. 1C) between 1.5 and 2.5 wk.

Plasma Haptoglobin and Albumin. Fig. 2A shows that plasma haptoglobin rose markedly after tumor implantation in three rabbits. In the same animals, there was little or no change in the concentration of albumin in plasma (Fig. 2B). Similar results were observed in five other rabbits.

Relationship of Plasma Ceruloplasmin to Plasma Prostaglandin Metabolites and Calcium. We have reported previously that PGE_2-M rises rapidly in the plasma of rabbits bearing the VX_2 carcinoma (8). The rise in PGE_2-M is much greater than the increase in PGE_2 itself (8, 11) and it precedes the elevation of plasma calcium concentration. The time-courses of changes in the concentrations of PGE_2-M, ceruloplasmin, and calcium in plasma in three rabbits bearing VX_2 carcinomas are shown in Fig. 3. In each rabbit, plasma PGE_2-M and ceruloplasmin rose earlier after tumor cell implantation than did plasma calcium. Plasma PGE_2-M and ceruloplasmin rose between wk 1 and 2, whereas plasma calcium did not rise above basal concentrations until 2–3 wk after tumor implantation (Fig. 3). These relationships are more clearly displayed when the results obtained in a group of rabbits are pooled and plotted together (Fig. 4). It is seen that the rises in plasma PGE_2-M and ceruloplasmin occur at approximately the same time, and that both precede the increase in plasma calcium. At 1 wk, plasma PGE_2-M was 340 ± 100 pg/ml (mean ± SE) as compared to a control value of 100 ± 30 pg/ml ($P < 0.05$), and plasma ceruloplasmin was 55 ± 5 mg/dl as compared to a control value of 37 ± 4 mg/dl ($P < 0.05$).

Effects of Indomethacin on Plasma Ceruloplasmin and Haptoglobin. The

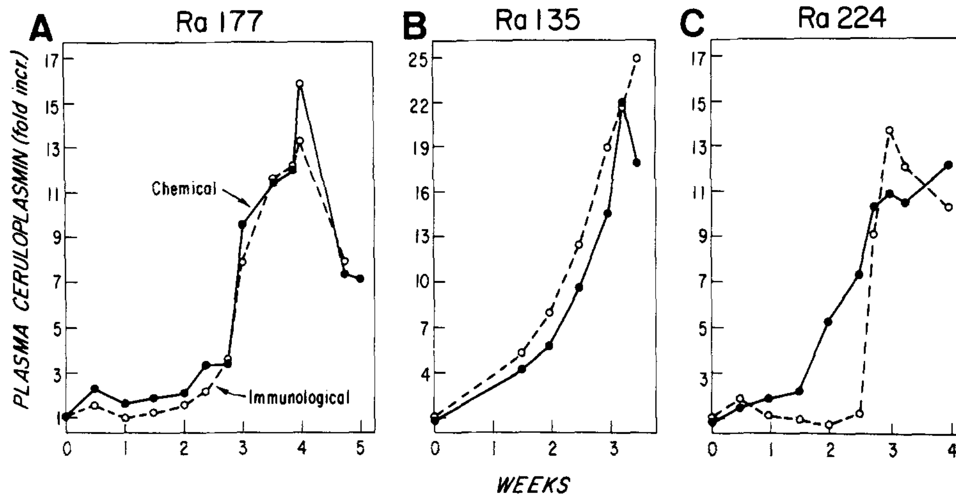

FIG. 1. Time-course of rise of plasma ceruloplasmin in three rabbits: (A), Ra 177; (B), Ra 135; and (C), Ra 224 after implantation of VX_2 tumor cells at 0 wk. The same plasma samples were assayed by the chemical (●——●) and immunological (○- - -○) assay procedures described in Materials and Methods. The samples for immunoassay were coded and ceruloplasmin was measured without knowledge of the experimental protocol. The data are plotted as fold increase (incr.) above the control basal value (set at 1.0) for each rabbit measured before tumor implantation.

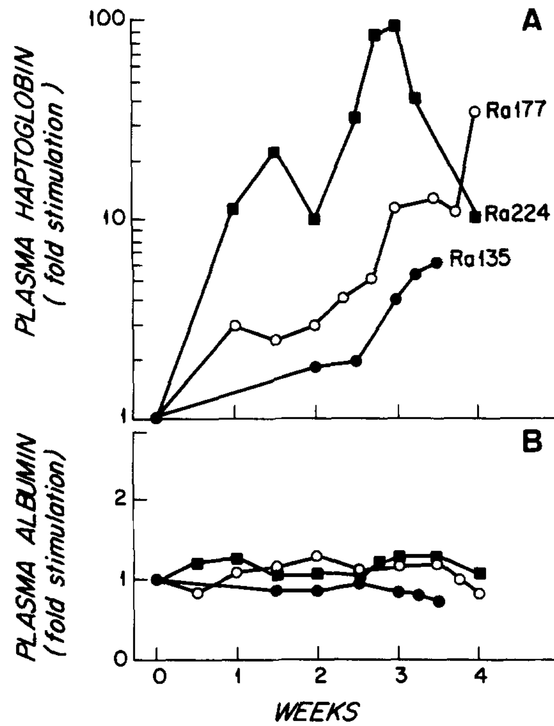

FIG. 2. Time-course of change in plasma haptoglobin (A) and plasma albumin (B) in three rabbits: Ra 135 (●), Ra 224 (■), and Ra 177 (○) after implantation of VX_2 tumor cells at 0 wk. The data are plotted as fold stimulation above the control basal value (set at 1.0) for each rabbit measured before tumor implantation.

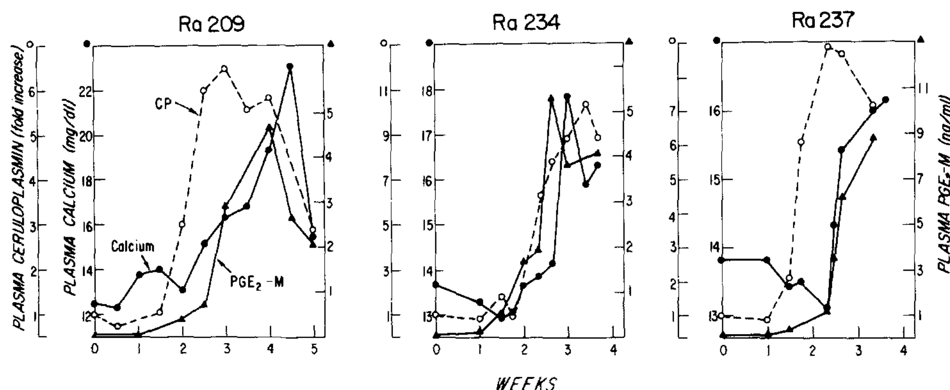

FIG. 3. Time-courses of rises of plasma PGE_2-M (▲——▲), ceruloplasmin (○- - -○), and calcium (●——●) in three rabbits (Ra 209, Ra 234, and Ra 237) after implantation of VX_2 tumor cells at 0 wk. The data for PGE_2-M and calcium are given as absolute concentrations in plasma, while those for ceruloplasmin (CP) are plotted as fold increase above the control basal value (set at 1.0) for each rabbit measured before tumor implantation.

anti-inflammatory drug indomethacin is a potent inhibitor of prostaglandin synthesis (29), and when administered to rabbits bearing the VX_2 carcinoma, it prevents the rise in plasma calcium (7), plasma PGE_2 (7), and plasma PGE_2-M (8), as well as decreasing the PGE_2 content of the tumor (7). Indomethacin also inhibits the synthesis of PGE_2 by strains of VX_2 tumor cells in culture (7).

In two rabbits, indomethacin administration was begun at the time of tumor cell implantation, and plasma calcium, ceruloplasmin, and haptoglobin concentrations were measured (Fig. 5). In contrast to the expected large rises in all three plasma components (Figs. 1–4), there was no increase in plasma calcium, and little or no change in plasma ceruloplasmin and haptoglobin (Fig. 5). If tumor-bearing rabbits were permitted to develop elevated concentrations of PGE_2-M and ceruloplasmin in plasma and were then treated with indomethacin, the continued rise in both plasma components was inhibited, or both components fell in parallel (Fig. 6). In Fig. 6A, temporary cessation of indomethacin administration was followed by an increase in plasma PGE_2-M and ceruloplasmin, both of which were decreased by a second course of treatment with indomethacin.

Discussion

From the results presented in this communication we conclude that the concentrations of the acute phase reactants, ceruloplasmin and haptoglobin, are elevated in the plasma of rabbits bearing the VX_2 carcinoma. The validity of this conclusion depends on the specificity of the findings and of the assay methods used. The clue that initiated our studies was the blue color of the plasma of tumor-bearing rabbits. Ceruloplasmin is a blue protein (17–19). The color of the rabbit plasma was not characteristic of bilirubin, the rabbits had neither hepatic metastases nor bile duct occlusion, and plasma bilirubin was not elevated (unpublished data). All plasma proteins were not elevated nonspecifically because plasma albumin remained unchanged (Fig. 2). Ceruloplasmin was measured in plasma by two independent techniques, a chemical method

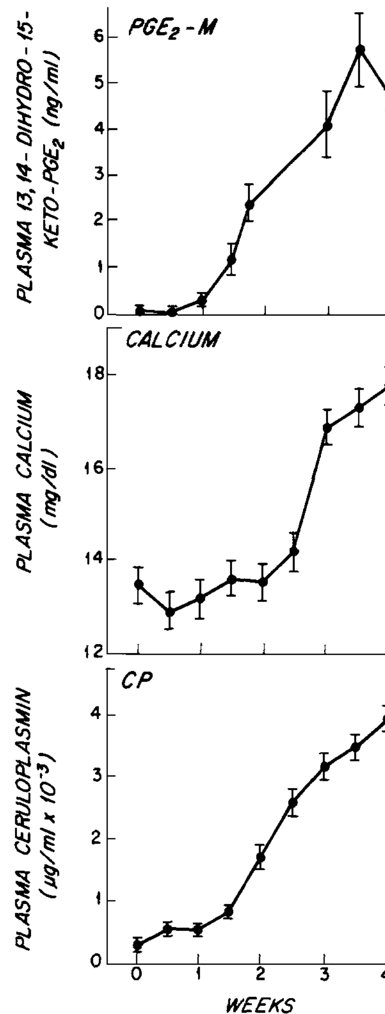

FIG. 4. Time-courses of changes in plasma PGE_2-M, calcium, and ceruloplasmin (CP) in a group of rabbits after implantation of VX_2 tumor cells at 0 wk. Each point gives the mean value of 7-12 rabbits and the bars give the SE. Rises above control values for PGE_2-M and ceruloplasmin were statistically significant ($P < 0.05$) at 1 wk and highly significant ($P < 0.001$) at 1.5 wk, whereas the rise in plasma calcium did not become statistically significant until 3 wk.

utilizing the *p*-phenylenediamine oxidase activity of ceruloplasmin, and an immunological assay, and the results of the two techniques were in good agreement. Furthermore, the subjective assessment of the increase in intensity of blue color in plasma correlated well with the results of both quantitative assay methods.

The time-courses of the rises in plasma ceruloplasmin and PGE_2-M in rabbits bearing the VX_2 carcinoma were very similar, and both clearly preceded the increase in plasma calcium (Fig. 4). We suggest the following hypothesis to explain our findings. The VX_2 tumor synthesizes and secretes large amounts of

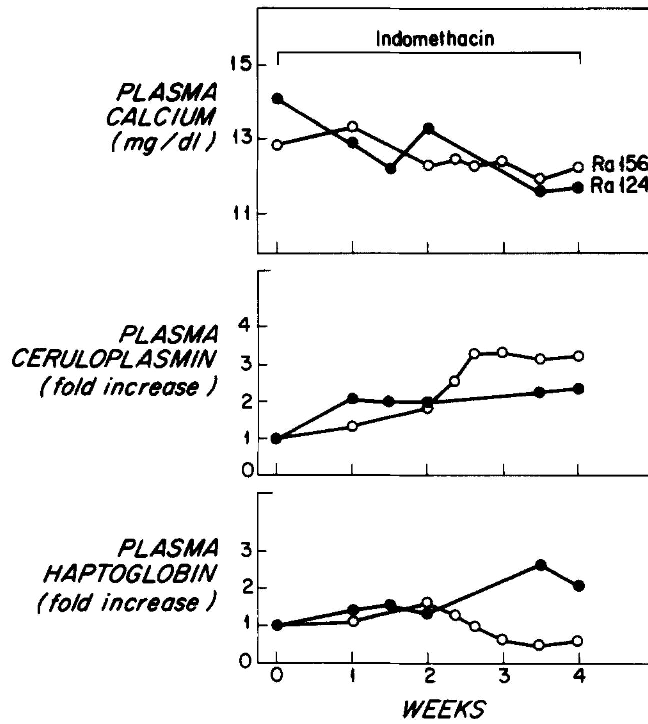

FIG. 5. Two rabbits, Ra 124 (●) and Ra 156 (○), were implanted with VX_2 tumor cells at 0 wk, and indomethacin (40 mg/rabbit/day, orally) therapy was begun immediately. Plasma calcium, ceruloplasmin, and haptoglobin were measured at intervals as described in Materials and Methods and plotted as described in the legends to previous figures.

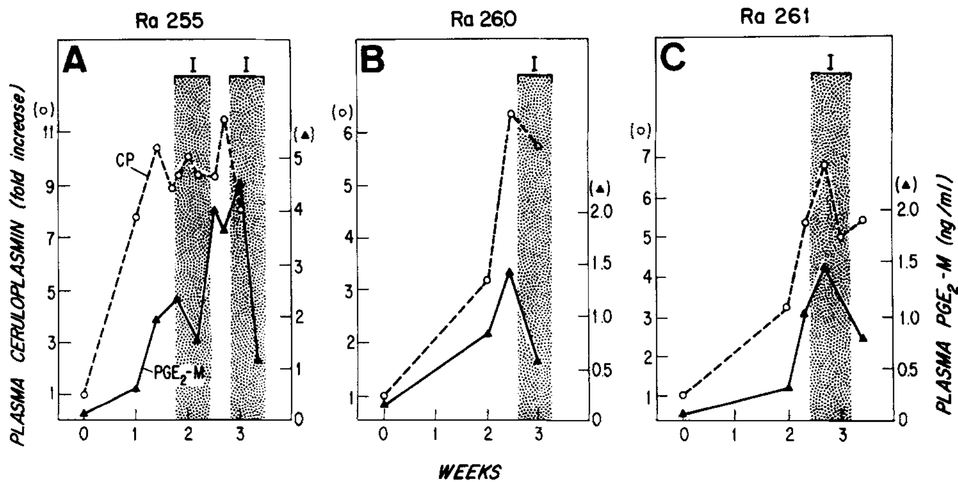

FIG. 6. Time-courses of rises of plasma PGE_2M (▲——▲) and ceruloplasmin (CP) (○- - -○) in three rabbits: (A), Ra 255; (B), Ra 260; and (C), Ra 261, after implantation of VX_2 tumor cells at 0 wk. The rabbits were treated intermittently with indomethacin (10–20 mg/rabbit/day). Indomethacin administration is indicated at the top of each panel by I over the shaded area. The data for plasma ceruloplasmin are plotted as fold increase above the control basal value (set at 1.0) for each rabbit measured before tumor implantation.

PGE_2 (7, 8, 11). Of the products of arachidonic acid metabolism secreted into plasma, PGE_2 is best measured as the accumulated metabolite, PGE_2-M because of the rapid clearance and metabolism of PGE_2 itself (8, 11, 12). One or more of these arachidonic acid metabolites, possibly PGE_2 or PGE_2-M, acts on the liver to stimulate the synthesis and secretion of ceruloplasmin (and also haptoglobin). This effect is more rapid and/or more sensitive to circulating arachidonate metabolites than is the action of PGE_2 on bone; thus the increase in plasma ceruloplasmin occurs before the hypercalcemia. We acknowledge that we have at this time no experimental evidence that the effect of the arachidonate metabolite is a direct action on the liver; it could be occurring indirectly via some additional mediator. Nevertheless, such a metabolite would appear to be a relevant intermediate in the pathway between tumor and hyperceruloplasminemia because its synthesis was inhibited by indomethacin, and there was little or no rise in plasma ceruloplasmin (or haptoglobin) in the presence of indomethacin. The validity of this interpretation depends on the assumption that the doses of indomethacin used did not have effects on ceruloplasmin (or haptoglobin) synthesis, release, or metabolism that are independent of the actions of the drug on prostaglandin synthesis. To our knowledge, no such effects of indomethacin have been reported, and no changes in plasma albumin concentrations were noted by us in rabbits treated with indomethacin.

The biological significance of our findings is of possible general interest. A body of evidence has accumulated that supports the view that a number of aspects of the inflammatory response are mediated via arachidonic acid metabolites and that the anti-inflammatory actions of aspirin-like drugs are due to their inhibitory effects on the fatty acid cyclooxygenase (30–32). In this context, the frequent association of elevated plasma concentrations of ceruloplasmin and haptoglobin with acute and chronic inflammatory processes is noteworthy. We have no evidence to suggest that in generalized inflammatory diseases the plasma concentrations of PGE_2, PGE_2 metabolites, or other metabolites of arachidonic acid are elevated, although the tissue levels may be high at localized sites of inflammation. On the other hand, inflammatory stimuli appear to enhance the synthesis and release of PGE_2, and possibly other metabolites of arachidonic acid, from macrophages (33). Thus it is possible that certain inflammatory stimuli lead to elevations in plasma of acute phase reactants, including ceruloplasmin and haptoglobin, via a pathway which depends on arachidonic acid metabolism. Consistent with this hypothesis is the observation that systemically administered PGE_1 causes a marked rise in serum haptoglobin in the rabbit (21).

In the case of tumors associated with elevations of plasma ceruloplasmin and haptoglobin, our findings indicate that, at least in the specific instance of the VX_2 carcinoma, the rise in these two acute phase reactants occurs in animals bearing a PGE_2-producing tumor. The large magnitude (10- to 20-fold) and early rise in plasma ceruloplasmin in rabbits carrying the VX_2 carcinoma indicate that this easily measured plasma protein may be used to monitor tumor presence and possibly the effects of anti-tumor therapy. Whether or not these observations could be extended to certain human tumors remains uncertain because of the probable multiplicity of factors controlling acute phase reactants in human subjects.

Summary

Results of previous studies have shown that the VX_2 carcinoma in rabbits synthesizes large amounts of prostaglandin E_2 (PGE_2). PGE_2 secreted by the tumor is rapidly metabolized and can be measured in plasma as the metabolite 13,14-dihydro-15-keto-PGE_2 (PGE_2-M). We have previously proposed that the hypercalcemia that occurs in rabbits bearing the VX_2 carcinoma is due to excessive secretion of PGE_2 by the tumor and its subsequent action on the skeleton as a bone resorption-stimulating factor. In the course of these studies, we noted that the plasma of rabbits bearing the VX_2 carcinoma became blue about 1 wk after tumor implantation. The intensity of the color increased markedly thereafter. We therefore measured ceruloplasmin in plasma by both chemical and immunological assay methods. Plasma ceruloplasmin and PGE_2-M rose in parallel (within 7–10 days) and preceded by 7–10 days the development of hypercalcemia. 2 wk after tumor implantation, plasma PGE_2-M and ceruloplasmin had risen about 20- and 6-fold, respectively, while the rise in plasma calcium was just beginning. Indomethacin, an inhibitor of prostaglandin synthesis, given from the time of tumor implantation prevented completely the hypercalcemia and largely inhibited the rise in ceruloplasmin. When given after hyperprostaglandinemia had developed, indomethacin produced a fall in both PGE_2-M and ceruloplasmin. A rise in plasma haptoglobin concentrations similar to that seen for ceruloplasmin was also observed. No changes in plasma albumin concentrations occurred. We conclude that the acute phase reactants ceruloplasmin and haptoglobin rise rapidly in the plasma of rabbits bearing the VX_2 carcinoma, and that this increase is related to arachidonic acid metabolism in these animals. It is possible that arachidonic acid metabolites also play a role in the elevations of these two plasma proteins observed in certain patients with malignant tumors.

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