Ceruloplasmin as a Marker of Neoplastic Activity in Rabbits Bearing the VX-2 Carcinoma

Hanna Ungar-Waron, Anita Gluckman, Eilahu Spira, Mordechai Waron, and Ze’ev Trainin

Section of Immunology, Kimmel Veterinary Institute, Bet Dagan [H. U-W., A. G., E. S., Z. T.], and Department of Internal Medicine, Asaf Harofe Government Hospital, Tel-Aviv University Medical School, Zrifin [M. W.], Israel

ABSTRACT

The establishment of an experimental model with rabbits in which VX-2 carcinoma was implanted in the gastrocnemius muscle and subsequently successfully cured by a second tumor cell inoculation has been reported previously. Tumor growth and regression could be followed by manual palpation.

The changes in serum ceruloplasmin (CP, EC 1.10.3.2) levels of individual rabbits during tumor development and regression were followed. CP levels increased 4- to 8-fold of normal during the progression of the malignant process, often before tumors could be detected by palpation. With tumor regression CP levels returned to normal. When metastasis developed, the CP levels remained high. This phenomenon seems to be related to the VX-2 carcinoma, since CP levels in rabbits challenged with various antigens and suffering from induced multiple s.c. abscesses did not change significantly, while in pregnant rabbits CP levels increased up to at most 3-fold.

It is concluded that serum CP level can serve as a reliable biochemical marker of the activity of this malignant process. The practical application of this finding lies in the follow-up of malignant processes in humans and is now under investigation.

INTRODUCTION

The search for specific biochemical markers for the detection and clinical evaluation of neoplastic diseases has been the object of numerous investigations in the past, but thus far with only limited success.

Increased serum CP levels were reported in various physiological and pathological conditions such as pregnancy (7, 14), estrogen-treated women (1, 14), myocardial infarction (8), chronic infections (7), and rheumatoid arthritis (5, 14). Increased serum CP levels occur also in a variety of neoplastic diseases in humans (4, 6) and have also been reported in the VX-2 carcinoma implanted into bone in rabbits (2).

CP therefore evoked interest as a potential tumor marker but, with the exception of 1 clinical evaluation in Hodgkin’s disease (4), no well-controlled systematic experimental study in neoplastic diseases was performed.

The changes in CP levels in humans were significant when groups of sick people and normal ones were compared statistically. However, in individual cases serum CP level becomes meaningless because of the wide overlap of the range of CP levels in normal and pathological conditions.

The main problem that prevented a more thorough estimation of the value of CP as a clinical marker of neoplastic disease was the fact that there was no clinical or experimental model in which one could follow the changes in serum CP levels during the development or regression of a well-defined malignant tumor.

The VX-2 carcinoma of rabbits was the model we chose for the establishment of such a system, as we have previously reported (15). The successful induction of tumor growth and its subsequent regression were obtained through injections with malignant cells.

It is the aim of this study to evaluate whether serum CP levels may specifically indicate malignant activity of experimentally induced VX-2 carcinoma in rabbits.

MATERIALS AND METHODS

Chemicals. p-Phenylenediammonium dichloride was purchased from Merck AG, Darmstadt, Germany, and recrystallized as described by Henry et al. (3).

Bovine serum albumin, essentially fatty acid free, was obtained from Sigma Chemical Co., St. Louis, Mo.

Animals. Random-bred rabbits of both sexes, 2 months old and weighing 2.5 to 2.8 kg, were used throughout the experiments.

Tumor Origin. The VX-2 carcinoma is a squamous cell carcinoma derived from a virus-induced papilloma of rabbits. With repeated transmission of the tumor, the virus dependency disappeared (2). Our source of the VX-2 carcinoma was a tumor-bearing Angora rabbit (the generous gift of Dr. Galasko of the University of London). The tumor was transplanted by i.m. implantation of a tumor explant into the gastrocnemius muscles of random-bred rabbits. The induced tumors obtained served as the source of VX-2 carcinoma cells.

Injection of Malignant Cells. Preparation of living tumor cells was carried out as previously described (15). A known dosage of $10^6$ cells was used for each injection administered into the gastrocnemius muscle of the rabbit. When a second injection of cells was administered to the same rabbit, it was injected into the other hind leg of the animal.

Detection of Tumor Growth. This was done by manual palpation of the injected zone. Positive detection was obtained when tumor size reached a diameter of 1.0 to 1.5 cm, occurring usually in the 4th week of the experiment. Six weeks after the administration of a $10^6$ cell injection, the tumor reached an 8- to 10-cm diameter. Metastases were detected by autopsy.

Bleeding of Animals. The rabbits were bled at weekly

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intervals, and their sera were separated, centrifuged, and kept frozen at −16°C until analyzed.

**Determination of CP Levels in Rabbit Sera.** The CP levels in rabbit sera were determined according to the method of Henry et al. (3), by which the oxidase activity of the CP is measured through its action on p-phenylenediammonium dichloride and the formation of Wurster’s red, which is followed at 530 nm. The CP units used are as defined by Henry et al. (3).

**Induction of Tumor Growth and Its Regression through the Administration of VX-2 Malignant Cells.** Forty-four rabbits were given injections of living tumor cells into their gastrocnemius muscles, and the CP levels in their sera were determined over a period of 12 weeks. These rabbits were divided into 2 experimental groups: Group 1 received the initial dosage of 10^6 malignant cells; Group 2 was given a second injection of the same dosage of cells in the other hind leg, 4 weeks after the initial injection.

**Pregnant Rabbits.** These 8 rabbits (Group 3) were observed from the day of copulation and over the period of gestation until 1 week after delivery.

**Control Groups.** There were 10 normal rabbits. In another control group were 12 rabbits into which multiple i.d. administration of antigens, synthetic or native, were injected in complete Freund’s adjuvant. The antigens administered were bovine serum albumin, bovine H-chains derived from bovine IgG, and ovine trypsin inhibitor. These injections produced multiple skin abscesses.

**Statistical Analyses.** All data were statistically treated by analysis of variance (11). From each experimental group, 8 randomly chosen rabbits were selected for statistical evaluation. The differences between the means were analyzed by multiple range test (Student test; Newman-Keuls test) (11). There is no significant difference between means marked by the same letter (p ≤ 0.05).

**RESULTS**

**CP Levels in Group 1.** Eighteen of 19 rabbits in this group developed tumors. In 4 animals, spontaneous regression of the tumor occurred. The CP levels in the sera of these animals were determined at weekly intervals in parallel with the determination of tumor growth, as detected by manual palpation. Examples are given in Table 1. One can see that the CP content of the serum remains constant as long as the tumor remains undetected but increases as tumor develops, reaching an 8-fold increase in Rabbit 71; it remained constant until the animal died.

In Rabbit 72, spontaneous regression of the induced tumor occurred, and the increase and decrease of the CP level in its serum were parallel to the appearance and disappearance of the tumor. In those weeks (Weeks 5 and 11), when tumor presence could be doubted by means of manual palpation, CP levels were higher than normal.

**CP Levels in Group 2.** In this group, the animals were given second injections of 10^6 cells, 4 weeks after the first injection. Twelve of 22 rabbits showed tumor regression while 8 died as a result of the cancer.

The CP levels along the experiment in the serum of an animal (Rabbit 84) in which induced regression of the tumor was obtained is shown in Table 1. Here again we can see a concomitant rise and fall of the CP level with the appearance and disappearance of the tumor.

In 3 rabbits no tumors developed after 2 injections of tumor cells. CP levels in these animals remained in the normal range along the whole period of the experiment.

**CP Levels as Criteria for Presence of a VX-2 Carcinoma, in Absence of Manual Palpation Evidence.** In Table 1 an example is given of an animal (Rabbit 77) in which manual palpation detected tumor formation in the 6th week of the experiment, but subsequently the tumor failed to expand and local regression, as judged by palpation, occurred in the 10th week. CP level rose significantly 7 days before positive manual detection of the tumor was obtained and remained high afterwards. On autopsy in the 13th week, lung metastases were found.

Increasing CP levels were often observed before the tumors could be palpated, whereas palpable tumor growth occurring prior to CP rise was never observed.

**CP Levels in the Sera of Pregnant Rabbits (Group 3).** The CP levels in the sera of 8 pregnant rabbits were determined along the period of their pregnancy. Their individual patterns differ from each other, but in all cases there is a significant rise in the CP content of the serum in the second part of pregnancy. The peak might occur 18 days after copulation or on the day of delivery itself. After delivery it drops back to normal in the 5th week (Table 2).

As seen in Table 2 the maximal mean values in the CP levels obtained in pregnancy are significantly lower than those obtained in the VX-2-induced carcinoma.

Three rabbits that did not become pregnant in spite of copulation exhibited no change in their serum CP levels for 30 days afterwards.

**CP Levels in Rabbits Undergoing Various “Challenge” Processes.** In order to ascertain the reliability of the CP levels as indicators of the presence of the VX-2 carcinoma, and not as markers of some unspecific reactions, the serum CP levels of the control groups were checked. These did not change significantly over long periods of time, even after high titers of antibodies were obtained.

The mean CP values obtained in the different experimental groups along the period of the experiment are summarized in Table 2. In all rabbits bearing the VX-2 carcinoma, independently of the group in which they belong, CP levels increase as tumor is detected and ultimately causes death about the 6th week after the beginning of the experiment; alternatively, the CP levels drop back to normal as the tumor disappears.

The 4 animals of Group 1 in which spontaneous regression of the tumor occurred exhibited a CP level curve identical with curves for those animals of Group 2 in which induced regression was obtained. Likewise the 8 animals of Group 2, which received a second injection of cells but did not exhibit tumor regression and died, showed a CP level curve identical with that of animals in Group 1.

**Autopsy Results.** Autopsies were performed on all experimental animals. In all the animals that died as a result of tumor growth, metastases in lungs, lymph nodes, or abdominal cavity were found. Animals in which induced or spontaneous tumor regression occurred were sacrificed on the 15th week after the beginning of the experiment. No primary or metastatic tumor was detected in these animals.
Table 1
Representative CP levels in sera of 4 individual rabbits with the VX-2-induced carcinoma

<table>
<thead>
<tr>
<th>Time (wk) after administration of 10^6 living cells</th>
<th>Rabbit 71</th>
<th>Rabbit 72</th>
<th>Rabbit 84</th>
<th>Rabbit 77</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>320</td>
<td>440</td>
<td>280</td>
<td>670</td>
</tr>
<tr>
<td>1</td>
<td>340</td>
<td>460</td>
<td>300</td>
<td>690</td>
</tr>
<tr>
<td>2</td>
<td>340</td>
<td>460</td>
<td>340</td>
<td>610</td>
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<tr>
<td>3</td>
<td>1320</td>
<td>520</td>
<td>560</td>
<td>+</td>
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<tr>
<td>4</td>
<td>2000</td>
<td>550</td>
<td>1380</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>2640</td>
<td>1280</td>
<td>1600</td>
<td>2160</td>
</tr>
<tr>
<td>6</td>
<td>2680</td>
<td>2240</td>
<td>1400</td>
<td>3000</td>
</tr>
<tr>
<td>7</td>
<td>2840</td>
<td>800</td>
<td>2920</td>
<td>Died</td>
</tr>
<tr>
<td>9</td>
<td>1000</td>
<td>440</td>
<td>2420</td>
<td>+ (small)</td>
</tr>
<tr>
<td>11</td>
<td>500</td>
<td>350</td>
<td>2520</td>
<td>?</td>
</tr>
<tr>
<td>13</td>
<td>530</td>
<td>360</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>380</td>
<td>270</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Rabbit 71 died as a consequence of the tumor expansion, as determined by postmortem examination. In Rabbit 72, the tumor underwent spontaneous regression. In Rabbit 84, regression of the tumor was induced by a second injection of 10^6 cells, 4 weeks after the initial inoculation. Rabbit 77 died 12 weeks after the beginning of the experiment; although apparent local tumor regression had occurred, metastatic spread in the lungs was observed upon postmortem examination.

Table 2
CP levels in the sera of the experimental groups of rabbits bearing the VX-2 carcinoma after tumor inoculation as compared to pregnancy

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>0</th>
<th>3 wk</th>
<th>4 wk</th>
<th>5 wk</th>
<th>10 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>541^a</td>
<td>± 25^b</td>
<td>570^c</td>
<td>± 67</td>
<td>521^d</td>
</tr>
<tr>
<td>Group 1</td>
<td>8</td>
<td>520^a</td>
<td>± 53</td>
<td>1056^d</td>
<td>± 137</td>
<td>2090^d</td>
</tr>
<tr>
<td>Group 2</td>
<td>9</td>
<td>496^a</td>
<td>± 28</td>
<td>836^f</td>
<td>± 81</td>
<td>1844^f</td>
</tr>
<tr>
<td>Group 3 (pregnant)</td>
<td>8</td>
<td>388^a</td>
<td>± 40</td>
<td>716^f</td>
<td>± 63</td>
<td>696^f</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
^a, ^c, ^d Means with different superscripts are different (p ≤ 0.05).

DISCUSSION

The VX-2 carcinoma model described provides an excellent possibility for the evaluation of biochemical tumor markers. Our results show conclusively that serum CP activity can serve as a reliable and reproducible parameter of the activity of the malignant process in this experimental model. It proves also to be reasonably sensitive, sometimes rising before palpable changes at the tumor inoculation site can be detected. The results observed in Rabbit 77 (Table 1), in which local tumor regression occurred concurrently with metastatic lung spread of the tumor, indicate that the CP levels can serve as a marker of primary as well as metastatic tumor activity.

The changes in CP level in this experimental outlay cannot be attributed to so-called stress reactions, as has been previously implied (13), nor does it seem to be the result of a nonspecific cellular proliferation process, for which pregnancy can serve as a model. The greatest change observed during pregnancy, although significant when compared to control values, is of the same magnitude as the changes observed at the beginning of tumor evolution (third week) when palpation is usually negative. Thereafter (Table 2), there is a highly significant difference between CP levels in tumor-bearing animals and pregnant ones, which leaves no doubt about the specificity of the process.

The pathophysiological mechanism of the increase in CP activity is not known. It could be the result of enhanced synthesis by the liver, stimulated by humoral factors, or a product of the proliferating tumor cells themselves. The synthesis of a new, more active species of CP is an additional possibility that cannot be excluded. This problem is now under investigation by estimating in vitro CP activity in tissue culture media of VX-2 carcinoma cells.

The main conclusion of this study points toward its possible clinical application, not as a primary diagnostic tool but as a means of follow up of the efficacy of antineoplastic treatment and early detection of posttreatment recurrence in the individual patient. It seems reasonable to assume that at least some clinically encountered neoplasms behave similarly to the VX-2 carcinoma; therefore CP may possibly be added to the available clinical markers of cancer, like carcinoembryonic antigen in colonic cancers (12, 16), RNase in pancreatic cancer (9), and other available biochemical tests (10).

The promising results described above, as well as the simplicity of the test, justify further clinical surveys in neoplastic diseases in man.

REFERENCES

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