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Carcinoembryonic antigen in retinoblastoma

An immunohistochemical study

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Abstract. Pathological amounts of carcinoembryonic antigen (CEA) have earlier been reported in the plasma of patients with retinoblastoma, and it has been suggested that CEA determinations be used in the follow-up of treatment of these patients. In the present study, 47 retinoblastoma specimens from the years 1962–1982 were examined. These specimens represented different clinical and pathological tumour types. Colon adenocarcinomata positive for CEA were used as controls. The laboratory method was a highly sensitive immunohistochemical peroxidase-staining procedure. By this method, CEA was not found in any of the retinoblastomata examined. It is probable that retinoblastoma does not produce CEA, but in theory it may indirectly increase the CEA titre or, on the other hand, be fully independent of CEA. Only after this relationship has been thoroughly clarified can determinations of plasma CEA in patients with retinoblastoma be used in clinical work.

Introduction

Carcinoembryonic antigen (CEA) was first described by Gold and Freedman (1965a and b) as an oncofoetal glycoprotein of the human gastrointestinal tract. It is a structurally heterogeneous peripheral membrane protein which can be found in the blood circulation as well (Rogers 1976). Subsequently, CEA has also been demonstrated in healthy adults and in connection with numerous malignant and benign disorders, and there is no general agreement as to its possible clinical applications (Anonymous 1981). Determinations of plasma CEA titre have mostly been used for the post-operative follow-up of patients with adenocarcinoma of the colon (von Kleist 1977; Anonymous 1981).

Elevated plasma CEA titre has been associated with several intraocular metastases (Michelson et al. 1976a, 1977; Char and Christensen 1980; Denslow and Kielar 1978) and with primary malignant melanoma of the uveal tract (Michelson et al. 1976a, 1977; Breborowicz et al. 1978). Pathological plasma CEA concentrations, which appear to decrease in the course of treatment, have also been observed in some patients with retinoblastoma (Michelson et al. 1976b; Gerke et al. 1978; Minoda et al. 1981). In asymptomatic relatives of retinoblastoma patients plasma CEA titre may be elevated more often than normal (Felberg et al. 1976). It has been suggested that determinations of CEA be used in monitoring the therapy of patients with retinoblastoma (Michelson et al. 1976b).

The measurements of CEA in plasma can be considered reliable only when the associated tumour has been shown to produce CEA. Thus, we became interested in possible observations of the production of CEA by retinoblastoma. Only Michelson et al. (1976b) have conducted such a study and they were unable to find CEA in retinoblastoma tissue. After their work, Hsu et al. (1981a and b) have described a new immunohistochemical staining method based on the strong interaction between avidin and biotin. The sensitivity of this method is up to 40-fold compared with the peroxidase-antiperoxidase method formerly considered to be the most sensitive one (DeLellis et al. 1979). The purpose of our study was to clarify by this method whether retinoblastoma expresses CEA and thus might be causing the pathological plasma titres observed. The clarification of this relationship is of crucial importance in assessing the benefit of CEA determinations in the follow-up of treatment of retinoblastoma patients.

Material and methods

Histological specimens. In the Ophthalmic Pathology Laboratory, Department of Ophthalmology, Helsinki University Central Hospital, 59 retinoblastoma specimens from 52 patients have been examined in the years 1962–1982. Some of these specimens had already been fully sectioned, or sent from other hospitals as paraffin sections, but in 47 cases (44 patients) the formalin-fixed paraffin-embedded tissue specimen was still available. According to the information received by the pathologist, 63.6% (28 patients) of these retinoblastomata were unilateral and 36.4% (16 patients) bilateral. The specimen was an enucleated globe except in one case which represented an orbital recurrence. Sections (5 μm-thick) were cut from the specimens and mounted with egg white on glass slides.

For controls, docent Torsten Wahlström kindly supplied 4-μm-thick paraffin sections from two adenocarcinomata of the colon both known to be CEA-positive.

Immunohistochemical staining. The immunohistochemical staining for CEA was carried out using a commercial version (Vectastain ABC Kit, Rabbit IgG, Vector Laboratories, Burlingame, CA 94010) of the avidin-biotinylated peroxidase complex (ABC) method of Hsu et al. (1981a and b). The staining was done as kindly instructed by docent

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Table 1. Outline of the Vectastain ABC Kit immunoperoxidase method for demonstrating CEA in paraffin sections as used in this study. MC = moist chamber, T = temperature 37° C. See text for details

1.	Xylene		
2.	Graded ethanol series		
3.	Methanol Perhydrol	100.0 ml 1.6 ml	30 min
4.	PBS		3 × 10 min
5.	Normal goat serum		30 min MC
6.	Primary antiserum		60 min MCT
7.	PBS		3 × 10 min
8.	Secondary antiserum		30 min MCT
9.	PBS		3 × 10 min
10.	Avidin-biotinylated peroxidase complex		30 min MCT
11.	PBS		3 × 10 min
12.	Sodium acetate Acetic acid Distilled water	35.2 ml 14.8 ml 50.0 ml	
	Distilled water	100.0 ml	
	3-amino-9-ethyl-carbazole N,N-dimethyl-formamide	40.0 mg 12.0 ml	20 min
	Perhydrol	200.0 µl	
13.	Running tap water		5 min
14.	Kaiser's glyceryl gelatin		

Torsten Wahlström and it is schematically presented in Table 1.

The sections were heated to the melting point of paraffin and deparaffinized in xylene and graded ethanol series. Possible endogenous peroxidase activity was destroyed by treating the sections for 30 min in a solution containing 100.0 ml methanol and 1.6 ml 30% hydrogen peroxide (Perhydrol, E. Merck, Darmstadt). They were then washed for three 10-min changes in phosphate-buffered saline (PBS, pH 7.4). To reduce the non-immunologic binding of the primary antiserum, the sections were incubated with normal goat serum (Vectastain ABC Kit, diluted 1:50 with PBS) in a moist chamber for 30 min at room temperature.

The primary antiserum used was commercial (Rabbit Antihuman Carcinoembryonic Antigen A115, Lot 069A, DAKO Immunoglobulins a/s, Copenhagen), and it had been produced in rabbit against CEA purified from hepatic metastasis from an adenocarcinoma of the human colon, and absorbed with normal human serum as well as blood group antigens A, B and 0. In a preliminary staining, the best result was obtained by diluting the antiserum 1:1000 with PBS, to which normal goat serum had been added 1:100 to prevent adsorption on glass surfaces. The sections were incubated in duplicate in a moist chamber at 37° C for 60 min, using the dilutions 1:100 and 1:1000 of the primary antiserum, and washed in PBS.

The biotinylated secondary antiserum directed against rabbit immunoglobulins had been produced in goat (Vectastain ABC Kit, diluted 1:250 with PBS) and the incubation was carried out for 30 min as mentioned above. During the following PBS washing, ABC complex was prepared by pipetting 5.0 µl Reagent A (Avidin DH, Vectastain ABC Kit) and 5.0 µl Reagent B (Biotinylated Horse-radish Peroxidase, Vectastain ABC Kit) for every 0.8 ml PBS buffer and mixing thoroughly. The sections were incubated as described above for 30 min and washed in PBS. With avidin acting as a bridge, the biotinylated secondary antibody bound the peroxidase molecules in the ABC complex to the antigen site.

A buffer consisting of 35.2 ml 0.2 M sodium acetate, 14.8 ml 0.2 M acetic acid and 50.0 ml distilled water was prepared for the staining solution and titrated to pH 5.0 with sodium hydroxide, whereafter an additional 100.0 ml distilled water was added. The staining solution was activated by adding 40.0 mg 3-amino-9-ethylcarbazole (Sigma Chemical Company, St. Louis, Mo. 63178) dissolved in 12.0 ml N,N-dimethylformamide (Uvasol, E. Merck, Darmstadt) and 200.0 µl 30% hydrogen peroxide. The sections were stained for 20 min at room temperature, rinsed in running tap water for 5 min, and the cover-slips mounted with Kaiser's glyceryl gelatin. No counter-staining was used.

Results

Histopathological examination. In sections stained with haematoxylin and eosin, 52.2% of the retinoblastomata formed distinct Flexner-Wintersteiner rosettes and could be classified as differentiated (Table 2; Zimmerman 1980). The bilateral retinoblastomata were more often differentiated than the unilateral tumours. Of the retinoblastomata 21.7% infiltrated into the choroid and 10.9% grew into the optic nerve. The unilateral tumours infiltrated into the choroid more often than the bilateral ones, whereas invasion of the optic nerve was equally common for both types.

Immunohistochemical staining. The known colon adenocarcinomata controls showed strong positive reaction for CEA with the 1:100 and 1:1000 dilutions of the primary antiserum. Staining was localized to the surface and the cytoplasm of the adenoid cells as well as to the luminal material. With the 1:100 dilution of antiserum, background staining was significant. Omitting any one step in the staining procedure resulted in a negative reaction.

All of the 47 retinoblastomata examined showed negative staining for CEA. However, in almost all tumours isolated cells with positive staining were found, due to non-specific reaction. Additionally, collagen of the sclera, necrotic areas in the tumour and subretinal fluid could give diffuse positive reactions because of non-specific binding, but the colour reaction was slight. In the limbal region, the corneoconjunctival epithelial cells regularly showed strong positive staining with the 1:1000 dilution of the pri-

Table 2. Distribution of the intraocular retinoblastomata examined into different clinicopathological groups

	Total	Unilateral	Bilateral
Flexner-Wintersteiner rosettes	52.2%	42.9%	66.7%
Infiltration into the choroid	21.7%	28.6%	11.1%
Infiltration into the optic nerve	10.9%	10.7%	11.1%
	$N=46$	$N=28$	$N=18$

mary antiserum. The colour reaction was mainly localized to the older surface cells. There were no differences in the stainability between the 1:100 and 1:1000 dilutions.

Discussion

Three research groups have independently shown that in some patients with retinoblastoma the concentration of carcinoembryonic antigen in plasma has been elevated to pathological levels (Michelson et al. 1976b; Gerke et al. 1978; Minoda et al. 1981). In all these studies, the average CEA titre was also noted to decrease in the patients treated. Thus, plasma CEA titre behaved in the same way as in patients with adenocarcinoma of the colon (von Kleist 1977; Anonymous 1981). In clinical practice a pre-operatively elevated CEA titre has generally been considered reliable (Anonymous 1981). Slightly elevated plasma concentrations are not significant, because CEA titres ranging from 2.5–5.0 ng/ml are also related to many benign disorders (Loewenstein and Zamcheck 1978; Anonymous 1981). It should be noted, however, that determinations of plasma CEA titre are reliable only when the associated tumour has been shown to be positive for CEA (Goldenberg et al. 1976).

In the present study, we were unable to demonstrate CEA in the 47 retinoblastomata examined. The material was unselected and representative of the clinical and pathological forms of retinoblastoma. Presumably the inheritance of all bilateral retinoblastomata (36.4%) was autosomally dominant, and some of the unilateral cases have also to be considered hereditary (Vogel 1979). Of the retinoblastomata 52.2% was differentiated and formed Flexner-Wintersteiner rosettes, 21.7% infiltrated into the choroid and 10.9% into the optic nerve. These clinical and pathological variables had no correlation to stainability for CEA.

Our observations agree with those of Michelson et al. (1976b) who stained 17 retinoblastomata by the immunohistochemical peroxidase-antiperoxidase method, considered to be somewhat more insensitive than the ABC method we used (Hsu et al. 1981a and b). Though a pre-operatively elevated plasma CEA titre was related to at least four of these tumours, all showed negative staining. Michelson et al. (1976b) also approached this problem by culturing cells in vitro from three CEA-associated retinoblastomata and from retinoblastoma cell line Y 79 (Reid et al. 1974). CEA was not radioimmunologically demonstrable in the tissue culture medium. Furthermore, the workers grew retinoblastoma cells in the anterior chamber of a rabbit eye. CEA was not demonstrable in the rabbit blood circulation, even though the tumour had metastasized to the lungs and the liver. Our results together with these observations lean strongly towards the theory that retinoblastoma does not express carcinoembryonic antigen. They do not, however, agree with the observations mentioned above, according to which retinoblastoma can be associated with a pathological CEA titre in plasma (Michelson et al. 1976b; Gerke et al. 1978; Minoda et al. 1981).

We believe that this controversy cannot be due to the methods used (Table 3). The avidin-biotinylated peroxidase staining method can be concluded to be at present the most sensitive means to demonstrate antigenic structures in formalin-fixed paraffin-embedded tissue sections (DeLellis et al. 1979; Hsu et al. 1981a and b). Some deterioration of antigenic activity is always related to formalin fixation, but the high sensitivity of the current immunoperoxidase methods compensates for this loss (DeLellis et al. 1979). Moreover, CEA is resistant at least to fixation in 10% formalin, and CEA activity may be retained for up to 35 years in formalin-fixed paraffin-embedded tissue (Harrowe and Taylor 1981). Differences between anti-CEA sera have been regarded as an important factor in CEA research (Tomita et al. 1974; Vrba et al. 1975). In our study, we used a known commercial primary antiserum which we, against the manufacturer's instructions, did not absorb with corresponding normal tissue. Thus, a false-positive result could be considered much more likely than a false-negative result. Michelson et al. (1976b) have excluded the possibility of a blocking CEA autoantibody. If CEA is present in retinoblastoma its amount has to be very small. Considering the inevitably small volume and the intraocular localization of retinoblastoma, it appears very unlikely that it could directly cause the pathological plasma CEA titres.

Table 3. Different models for explaining the relationship between retinoblastoma and carcinoembryonic antigen

Presuming retinoblastoma produces CEA
1. The method of demonstration is too insensitive
2. CEA is destroyed during tissue preparation
3. Difficulties due to immunologic heterogeneity of CEA and antisera
4. CEA is covered by some other structure
Presuming retinoblastoma causes elevated plasma CEA titres indirectly
1. Synthesis of CEA elsewhere in the body is induced by: interferon; unknown factor
2. CEA metabolism is transformed
3. CEA production by tumours associated to hereditary retinoblastoma
Presuming retinoblastoma is a phenomenon separate from elevated plasma CEA titres
1. Plasma CEA titres are physiologically higher in children than in adults
2. Plasma CEA titre elevated due to some other intercurrent disorder

There are, however, various other possibilities to explain the well-documented association between retinoblastoma and pathological plasma CEA levels (Table 3), though at the moment none of these theories can be considered preferable to the others. On the basis of our own observations and those of Michelson et al. (1976b) we must question the value of plasma CEA determinations in monitoring the treatment of patients with retinoblastoma until a more thorough knowledge of the tumour-CEA relationship has been achieved. In particular, further research is required into the normal distribution of plasma CEA titres in healthy children.

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