Vol. 163, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1989 Pages 635-641

CHARACTERIZATION OF CASEIN KINASE II IN HUMAN COLONIC CARCINOMAS AFTER HETEROTRANSPLANTATION INTO NUDE MICE

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Received July 4, 1989

Casein kinase II (CKII) activity in colorectal tumours was compared before and after heterotransplantation onto nude mice. The test revealed that the enzyme activity was about two-fold enhanced in the tumours isolated from the nude mice when compared to the respective primary tumours from which they were derived. Immunoblots using a polyclonal CKII-specific antibody showed that the increase of activity was due to a higher expression of the enzyme. Immunohistochemical studies on cross sections of nude mouse tumours showed that most of the CKII molecules were located at the peripheral part of the tumour; the central part did not show intense CKII-specific staining.

It is now well established that casein kinase II (CKII) is closely associated with proliferation. This has been shown in rapidly growing tissue cultures (1,2, Schneider and Issinger, 1989 submitted for publication) in mitogen-stimulated lymphocytes (3), during certain stages of embryogenesis (4), during progesterone-stimulated maturation of oocytes (5), in solid tumours and in cells at the basis of colon villi, which are known for high mitotic activity (Münstermann et al.,1989 submitted for publication). All this evidence together with observations that CKII phosphorylates preferably proteins associated with rDNA transcription (6-10) and translation (11-15) suggest a pleiotropic function for this protein kinase during the course of proliferation. Here we show that CKII which is already overexpressed in solid human tumours is further enhanced after heterotransplantation into nude mice. This increase in enzyme activity is due to an increase of CKII molecules as could be shown by immunodetection with a polyclonal anti-CKII antibody. Furthermore it is shown by immunohistochemical studies that most of the enzyme is located at the peripheral actively proliferating part of the tumour.

So far mitotic activity is commonly determined by ^{3}H-thymidine labelling or by application of the proliferation marker Ki67, a monoclonal antibody against a so far uncharacterized nuclear antigen (16). Both methods have certain disadvantages: ^{3}H-thymidine labelling is a tedious and laborious method with all the

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0006-291X/89 $1.50

problems involved of in vitro labelling tissue slices and subsequent detection of labelled DNA. Immunodetection of Ki67 on the one hand is very specific, since it only shows positive signals in the nuclei of proliferating cells. On the other hand Ki67 cannot be used for biochemical studies since it is devoid of enzyme activity. Furthermore Ki67 cannot be detected by Western blotting and subsequent immunostaining (for so far unknown reasons) and thus does not allow a quantitative determination of this proliferation-specific antigen.
CKII has the advantage that it can be characterized by its enzyme activity and it is also readily detectable by immunohistochemistry in tissue slices and cells. However, since the enzyme is always present in all cells, in the cytoplasma as well as in the nucleus, rapidly proliferating cells can only be detected by an increase of CKII (either by activity measurement or immunodetection) with the obvious disadvantage that there is always a natural background staining resulting from the cells that are not in a proliferative state.

MATERIAL AND METHODS

Biological material- Tumours were obtained from the local pathology department fresh after surgery. The material was either used directly or from liquid nitrogen stocks. Tumours were classified according to the actual TMN classification (17). The colonic carcinomas used throughout this study were usually moderately differentiated.

Heterotransplantation into nude mice (Balb/c/nu/nu)- Tumours were cut in 3 x 3 x 1 mm pieces and transplanted into a skin pocket above the forleg. Tumours were harvested 3-4 months after transplantation.

Enzymes and antibodies- Casein kinase II (CKII) was isolated from hog spleen as described previously (7). Antibodies to hog spleen casein kinase II were generated in rabbits (Münstermann et al., 1989 submitted for publication).

Preparation of cell extracts- About 0.1 to 0.5 g of tissue were minced and then transferred to 0.5 to 2 ml of ice-cold buffer A (0.25 M sucrose, 30 mM Tris-Cl⁻ pH 7.4, 1 mM EDTA). The material was then homogenized with an Ultra-turrax (20,000 rpm) three times for 30 sec at 30 sec intervals. The homogenate was sonicated four times for 15 sec at 10 sec intervals at 50 Watt using a Branson sonifier with a microtip. The sonicated material was then centrifuged at 15,000 x g at 4°C for 20 min in a Beckman centrifuge. The protein content was determined and the concentration adjusted to 1 mg/ml with buffer A. This extract was used for the CKII activity test.

Casein kinase II activity- CKII activity was determined as described earlier (4). The reaction was carried out in the presence of 20 mM Mes-OH⁻ (pH 6.9), 130 mM KCl, 10 mM $MgCl_2$, 5 mM DTE, 0.5 % casein (dephosphorylated), 50 µM ATP. The specific activity of (τ-^{32}P)ATP was 100-500 cpm·pmol^{-1}. 30 µl from a total 50 µl reaction mix were removed and spotted onto Whatman 3 MM filters. TCA precipitation and liquid scintillation counting was as described previously (4).

Immunodetection of CKII- When not otherwise specified, 200 µg of protein were applied per gel and processed as described earlier (18).

Immunohistochemical staining of CKII- Based on the study of Hancock (19), we used a modified sequential application of the avidin-biotin-complex (ABC-technique) with nickel intensified diaminobenzidine (DAB) for immunohistochemical staining of CKII.

RESULTS AND DISCUSSION

Here we show that CKII activity is enhanced in colorectal carcinomas after heterotransplantation into nude mice. Earlier investigations (Münstermann et al.(1989), submitted for publication) had shown that CKII is generally enhanced by a factor of 2 in colorectal carcinomas when compared to the non-neoplastic tissue. Almost all of the tumours investigated were classified as moderately differentiated. An analysis of 18 primary carcinomas showed that CKII activity could be further enhanced by a factor of 2 when passaged through nude mice (table 1). The increase in CKII activity was statistically significant as shown using Student's t-test (two-tailed) with a significance level of 0.01% ($t = 4.22134$; $t_{17;0.001} = 3.965$) (table 1). Since CKII expression is associated with proliferation (1-15), the data are in agreement with the finding that tumours, when transplanted into nude mice, exhibit a significant higher mitotic activity than the original tumour (20). This can be explained by a better vascularization and a better nutritional environment in the nu/nu mouse.

The CKII activities measured in the primary colorectal carcinomas were in the range of 20-432 U/mg protein. However, most of the tumours exhibited a CKII activity between 100-300 U/mg protein.

It is well known that CKII activity is strongly stimulated by polyamines (spermine and spermidine) (21) and that ODCase plays a central role in regulating

Table 1: **CKII activity from 18 primary colorectal carcinomas and the corresponding heterotransplants after passaging through nude mice**

TUMOUR NO.	HETEROTRANSPLANTED TUMOUR	PRIMARY TUMOUR	% INCREASE (HT/PT)
H 13712/87	170	128	33
H 2087/87	164	78	110
H 1958/87	262	192	36
H 2253/88	244	166	47
H 7968/87	514	432	19
H 2784/88	276	286	-
H 4674/87	176	88	100
H 14172/87	190	150	27
H 8007/87	390	246	59
H 8155/87	172	78	121
H 7428/87	278	44	532
H 3519/87	162	130	25
H 12749/87	172	150	15
H 7601/88	236	184	28
H 12571/88	186	288	-
H 680/87	468	120	290
H 2393/87	82	20	310
H 12714/88	444	282	57

CKII activity was expressed in U/mg protein. 1 Unit corresponds to the transfer of 1 pmol ^{32}P into TCA-precipitable material at 37°C in 1 min. Percent increase of CKII-activity in the tumours was calculated from the ratio of heterotransplanted vs. primary tumours. The H-numbers are internal case numbers. Student's t-test (two-tailed) showed a significant increase of CKII activity ($t=4.22134$; $t_{17\ 0.001} = 3.965$) in the heterotransplanted tumours when compared to the primary carcinomas from which they were derived.

polyamine synthesis. In this way our results may be seen as an extension of investigations where it was shown that ODC activity is overexpressed in cases of familial polyposis (22). Increased ODCase activity is a requirement for the onset of proliferation in intestinal mucosa (23). Furthermore polyamines have been studied as potential markers of neoplastic diseases (24) including colorectal carcinoma (25). In the case of familial polyposis, patients with different kinds of polyps expressed different ODC activities. The observed variation in ODC activity was in a range of +/- 200 U. Taking all these facts together, the observed CKII increase in colonic tumours may well be mediated by an initial increase of ODCase and the resulting higher polyamine levels in the cell.

We can only speculate that the tumours were derived from different parts of the colon and therefore possess already an inherent higher basal CKII activity. That this could be indeed the case was shown by Traffa et al. (unpublished results) who found a gradient of CKII activity when screening various parts of the colon. Further support comes from observations where ODC activity was shown to be higher in left-sided cancer cases than in right-sided cancer cases from large bowel cancers (26).

In order to show that the enhanced CKII activity was due to an increase of CKII molecules, immunoblots with CKII-specific antibodies were carried out.

In order to give a visual impression of the results obtained, fig.1A shows the CKII activity from tumours #H2253/88 and #H7968/87 before (PT) and after heterotransplantation onto nude mice (NM).

The original tumour H2253/88 belongs to a group of tumours with average CKII activity (fig.1A, table 1) (160 U/mg protein). After heterotransplantation it showed an activity of 244 U/mg protein which accounts for an activity increase of 47% (table 1).

The second tumour, shown in fig.1A, H7968/87 (PT), exhibits a higher CKII activity (432 U/mg protein) than tumour #2253/87 (fig.1A, table 1). The increase in CKII activity after passaging through the nude mice was 19% (table 1). The differences in percent increase of CKII activity after heterotransplantation onto nude mice can be explained by the varying amount of necrotic tissue which is always present to a large extent in these fast growing tumour transplants.

Fig.1B shows the immunoblots from the primary tumours (PT) and the heterotransplanted tumours (NM), H2253/88 and H7968/97, respectively. The activity ratios are reflected in the signal intensity obtained after immunoblotting of tumour extracts and detection by an anti-CKII-specific antibody. Tumour H2253/88 with a CKII activity of 244(NM)/166(PT) U/mg yields a weaker immunostaining pattern than tumour #H7968/87 (541(NM)/432(PT) U/mg) due to its lower CKII activity (fig.1B).

The amount of CKII in the tumours was determined by using a calibration curve where different quantities of purified casein kinase II were applied to the gel, blotted to nitrocellulose filter and detected by immunostaining with an anti-

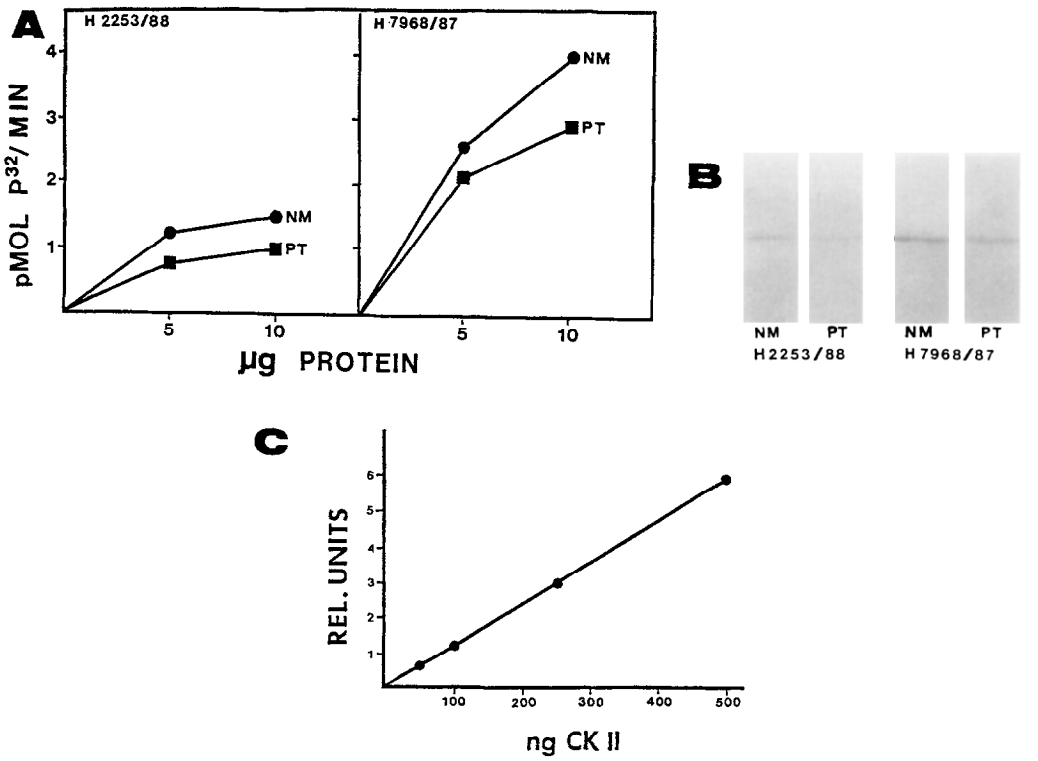

Fig.1: **(A) CKII activity from primary colon tumours (PT) and after heterotransplantation onto nude mice (NM). (B) Immunoblots of colonic tumour extracts before and after heterotransplantation onto nude mice.** 200 µg of extracts were applied to the gel. (C) Calibration curve based on the amount of purified CKII (ng) and the signal intensity obtained after reaction with with an anti-CKII-antibody on the immunoblot. The signal intensity in tumour # H7968/87 (NM) corresponds to roughly 40 ng CKII. Given a mol. mass of 130 kDa the amount of CKII in 1 g of tumour tisue amounts to about 1.5 nMol.

CKII antibody (fig.1C). However, care must be taken in using immunoblots for exact quantitative determinations of the CKII content in cells since the method has its limitations, especially when lower amounts of protein are detected (<100 ng).

The amount of CKII in the tumours investigated was in a range of 0.8-1.5 µMol/kg tumour.

When tumours were removed from the nude mice, 3 mm thick slices were prepared. Cross sections from the frozen slices were prepared and stained to detect CKII. Fig.2 shows a cross section through a colorectal carcinoma after passaging through nude mice. It can be seen that most of the CKII is located at the peripheral zone of the tumour (A) whereas CKII molecules are sparse at the central part of the tumour (B). This observation can be easily explained. The fast growing tumours in nude mice usually consist of a necrotic core with only a relatively thin peripheral growth zone of higher proliferative activity.

This result is well in agreement with data (27) where it was shown by ^{3}H-thymidine labeling that differentiated colon carcinomas exhibit a gradient of pro-

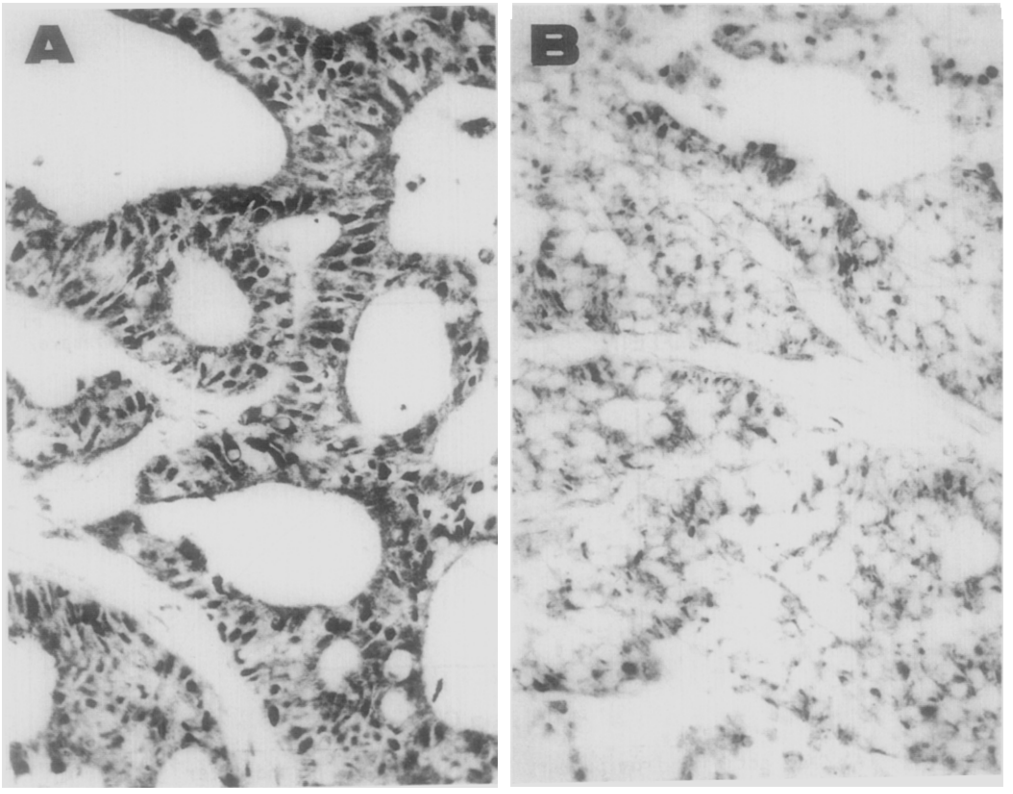

Fig.2: **Immunostaining with an anti-CKII antibody of a cross section through a colonic tumour after heterotransplantation onto nude mice.** (A) Peripheral part of the tumour; (B) Central part of the tumour. Immunostaining was as described in the material and method's section. The presence of CKII is indicated by the black staining of the nuclei. The staining intensity (also expressed by the size of the stained nuclei) is correlated with the amount of CKII present in the individual tissue area. Magnification of the cross section was 360-fold.

liferative activity which is increasing from the basis to the surface of the tumour. This also correlates well with the dominant growth direction of these tumours in the colon lumen.

The findings described here show that CKII expression can be artificially enhanced by heterotransplantation of colorectal carcinomas in nude mice. This gives further support to the hypothesis that CKII expression is closely associated with proliferation.

ACKNOWLEDGMENTS

We would like to thank Dr. B. Boldyreff for critically reading the manuscript. This work was supported by SFB grant 246/B3 to O.-G.I.

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