The Histochemical Observation of the Experimental Carcinogenesis on the Mouse Skin

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Introduction

Experimental carcinogenesis of the skin has been carried out by many investigators, but there are only a few with histochemical observations. BieseI, Kidd, Winzler, and Burk reporting on the histochemical demonstration of ALKp-ase noted that very intense activity of the enzyme was seen both in the stroma and epidermal cells, but of less activity in cells far from the stroma. Morreti and Mescon, Rutenburg and Seligman noted the localization and distribution of ACDp-ase in epidermal tissue, and Greenstein, Gomori, Cohen,
Nachlas, and Seligman surveyed the activity of esterase. The histochemical demonstration of glycosidase in tumors was described by Braun-Falco, Monis and Rutenburg, and the distribution and localization of aminopeptidase by Burstone, Monis, and Seligman. Eisen, Montagna and Chase, Hayashi, Hashiguchi et al. reported on histochemical observations on protein bound sulphydryl and disulfide groups. But little work has been done to study several enzymes comparatively at the same time on their localization and distribution. Therefore, in this study the histochemical changes of several enzymes in mouse skin in experimental carcinogenesis were examined, and compared in localization and distribution to each other.

**Materials and Methods**

One hundred mice of both sexes were used for the experiment. A 0.5% solution of 20-methylcholanthrene in acetone was applied to the shaved skin twice a week continuously during 20 weeks. Six animals each were sacrificed every 2 weeks, and carcinogenetic process was observed pathologically and histochemically on the several stages. Fresh-frozen sections (10-20μ) were made from such materials in the cryostat at −20°C and histochemical demonstration of enzymatic activity and protein bound sulphydryl and disulfide groups during carcinogenic changes of the skin were carried out.

The methods of histochemical demonstration which were employed for ALKp-ase, ACDp-ase, esterase, β-glucuronidase, β-galactosidase, β-glucosidase, aminopeptidase, protein bound sulphydryl and disulfide groups, polysaccharide, are shown in table I. Several parallel sections were stained with H. E. and reacted by each enzymatic histochemical technique. In the same material, after fixation in 80% ethyl alcohol, paraffin sections were examined for the demonstration of the polysaccharide with one specimen, and another specimen was stained with H. E. for histological diagnosis on several stages.

**Results**

Normal skin: ALKp-ase activity was localized in capillaries and hair fol-
licles, and no activity was observed in epithelium. The localization of ACDp-ase and esterase was in whole epithelial cells and hair follicles, but ACDp-ase reacted more intensely in the cornified layer and granular layer, and strong activity of esterase was contained in the granular layer. Aminopeptidase was observed slightly in the basal cell layer of the epithelium and moderately in hair follicles. β-galactosidase reaction was present slightly in epidermis, and intensely in hair follicles and subcutaneous fat tissue. β-glucuronidase and β-glucosidase were located similarly to the localization of β-galactosidase. The distribution of protein bound sulfhydryl and disulfide groups was mostly similar to that of ACDp-ase. No PAS positive polysaccharides were found in the epithelial cells of normal skin.

In 3rd week: Epithelium was slightly hyperplastic and the cornified layer of the skin increased in thickness as a whole. Hair follicles were mostly destroyed or diminished. Basal cells of the epithelium had begun to proliferate invading the subcutaneous connective tissue, and some of the rete pegs were elongated downwards. No inflammatory cells were noted in connective tissue which was also a little hyperplastic. The sites of ALKp-ase activity were similar to normal skin and not appeared in subcutaneous connective tissue. The localization of ACDp-ase and esterase activity, in this stage, were the same as normal skin. Aminopeptidase activity appeared moderately in the basal cell layer of epithelium which proliferated downwards invasively, and in the subcutaneous tissue adjacent to the invasively proliferating epithelium and elongating rete pegs. Mast cells infiltrated in subcutaneous connective tissue near the hyperplastic and downgrowing epithelium, and they also showed a positive reaction for aminopeptidase. The protein bound sulfhydryl and disulfide groups were abundant especially in the cornified and granular cell layer. With PAS reaction no polysaccharides were observed in epidermal and dermal tissues as in the normal skin.

In 8th week: Hyperplasia and invasive proliferation of the epithelium were promoted. The partly destroyed or irregular basal membrane and irregular cell arrangement of the basal cell layer were noticed and they demonstrated an increased rate of proliferation. Mast cell infiltration and fibrous hyperplasia in the dermis were observed more intensely than in the early stage, especially in the part adjacent to proliferating epithelium. In this region, strong ALKp-ase activity was observed. Aminopeptidase activity was not changed as in the 3rd week, but the reaction was increased in the ground substance of connective tissue and in mast cells. In these findings, it is very interesting that the sites of the enzymatic activity in the basal cell layer were always adjacent to those in the dermis. No histochemical changes of the localization of sulfhydryl groups, disulfide groups and other enzymes were found, and also PAS positive polysaccharides were not contained in the epithelium.

In 12th week: Carcinogen-treated epithelium showed a more advanced stage of hyperplasia. In this stage, piknosis and karyolexis of the nucleus, irregular form and disarrangement of the cells in some regions of experimental carcinoma were noted. These findings showed the malignancy of hype-
rplastic proliferation, and on these facts the experimental tumorigenesis could be diagnosed as cancer. The histochemical reaction of all the enzymes examined showed generally little changes in comparison with those in the 8th week. But, tumor epithelium was thickened, each layer of epithelium was apparently defined and the localization of enzymes was observed more significantly. ACDp-ase, protein bound sulfhydryl and disulfide groups were abundant mainly in the cornified layer and in the granular cell layer. Esterase was contained in the granular layer, and glycosidase in the granular layer.

In 16th week: The experimental carcinogenesis was further promoted. Carcinoma pearl formation was rich, and the majority of tumors were diagnosed as the squamous cell carcinoma. The tumor cells invaded through the muscular layer into the subcutaneous tissue. The carcinomatous proliferation was already found in the remained animals, however, there were no alterations in the histochemical findings of ACDp-ase, esterase, glycosidase, polysaccharide, protein bound sulfhydryl and disulfide groups, but ALKp-ase activity of the carcinoma tissue disappeared from adjacent connective tissue except for some capillaries. Aminopeptidase activity was also lesser, but slightly in the connective tissue adjacent to the tumor cells and in the infiltrated mast cells which decreased in number.

Discussion

The histochemical changes of various enzymes in the skin on carcinogenic process induced by 20-methylcholanthrene were most marked in the ALKp-ase and the aminopeptidase. The appearance of ALKp-ase activity was observed in the connective tissue adjacent to the proliferating epithelium, when the epithelium began to proliferate invasively into the subcutaneous connective tissue, and ALKp-ase activity became more intense in accordance with the high epithelial proliferation. Many investigators reported that ALKp-ase activity in the normal skin was localized in capillaries and hair-follicles, and not in the epithelial cells. It is sure from some experiments that ALKp-ase was found in the fibroblastic parts of the skin, and usually in the regions of chronic inflammation. In histochemical studies of wound healing of the epithelium, it was observed that ALKp-ase activity appeared in immature granulations which played a part of repairing the defect of injured skin as regenerating tissue. ALKp-ase was strikingly demonstrated in the infiltrated inflammatory cells in most cases of wound epithelium and in necrotic parts of epithelium. These findings demonstrated that ALKp-ase activity appeared in the tissue undergoing not only fibroblastic regeneration but also destruction. Several investigators reported that ALKp-ase was abundant in the connective tissue adjacent to the tumor in experimental carcinoma and other tumors. Biesele reported that ALKp-ase was abundant in the basal cells in direct proportion to proximity of positive dermal element and very intense both in the stroma and epidermal cells adjacent to the stroma. But from the present experiments it was shown that these conclusions were not correct, and the ALKp-ase activity was never marked in the epithelial cells on whole pro-
cesses of carcinogenesis. Biesele and others reported that ALKp-ase was strongly reactive in the connective tissue surrounding the carcinized epithelium. But, in the present experiment, it was observed that ALKp-ase activity was lessened or diminished in the connective tissue, except for the capillaries and hair follicles, as the tumor induced by 20-methylcholanthrene grew to maintain epithelial pearles. It was not decided whether these findings demonstrated that the tumor stopped growing or lessened the growth-rate, or that the surrounding connective tissue lost the stromal reaction or the resistibility for the tumor invasion.

Aminopeptidase appeared in the connective tissue adjacent to the invasively proliferating epithelium, as the epithelium began to proliferate invasively into the subcutaneous connective tissue, and the enzymatic activity was intense in the ground substance of the connective tissue in hyperplasia. Mast cells infiltrated into the adjacent connective tissue and showed a positive reaction of aminopeptidase activity.

In 1956, Burstone first described the histochemical demonstration of prominent aminopeptidase activity in the connective tissue adjacent to invading tumor and in the inflammatory tissues, and showed that the aminopeptidase activity was strong in the connective tissue adjacent to the tumor and in the tissues with chronic inflammatory changes, especially in the ground substance, infiltrated inflammatory cells and necrotized parts of the tissue. Subsequent validations of these findings had been reported by Braun-Falco, Willighagen, Monis, Nachlas, Seligman, Glenner, Burstone, Meyer, Mori et al, and Wattenberg. These investigators reported that, most of neoplastic cells were generally free of aminopeptidase, but, basal cell carcinoma had a little aminopeptidase activity in the tumor parenchyma. This finding demonstrated that the localization of aminopeptidase activity in the carcinoma was similar to those in the matured normal epitheliuma. There are two major opinions about the function of aminopeptidase. One was pronounced by Glenner, Burstone and Meyer that the activity was correlated with the invasive capacity of the tumor, but was not related to the grade of anaplasia or the extent of adjacent fibroplasia. They further stated in their reports that aminopeptidase activity in the adjacent stroma was evidence of proteolysis and suggested that a prominent mechanism of tumor invasion was proteolytic destruction of the stromal component, and that aminopeptidase was involved in proteolysis rather than synthesis, its activity being also indicative of proteolysis. The other was published by Monis, Nachlas, and Seligman in which they rejected the hypothesis of Burstone. They proposed that the aminopeptidase activity of the stroma was a biological property of proliferating connective tissue cells which had no special relationship to the invasive property of tumor, but had an inherent property of fibroblastic activity. According to several findings in this experiment and previous reports of histochemical observations in wound healing, these two different opinions about the function of aminopeptidase can both be considered. As observed in wound healing, aminopeptidase was abundant in the wound surface undergoing necrosis, and subsequently in fibro-
blastic connective tissue. It was suggested that the former demonstrated the proteolytic property and the latter fibroblastic. It is supposed that, in carcinogentic process, the destruction and reparation of the connective tissue was carried out simultaneously, both a proteolytic reaction and fibroblastic reaction took place in the same part of tissue, and that aminopeptidase activity demonstrated both properties of proteolysis and fibroplasia. ACDp-ase and protein bound sulfhydryl and disulfide groups were marked in the same portions of tissue of normal and carcinogenic skin. Both were abundant in the cornified layer and granular cell layer, and in epithelial pearls of squamous cell carcinoma. These findings were previously reported by many authors; i.e. on ACDp-ase by Moretti and Mescon3), Rutenburg19 and Mori et al21), and on protein bound sulfhydryl and disulfide groups by Hayashi14) and Deguchi et al18). Esterase was rich in the granular layer. This finding was coincident with reports of Cohen, Nachlas, Seligman15), and Mri et al21). On glycosidase, many investigators16,17,27,32,33) reported of normal skin and in malignant tumors that the enzymatic activity was strong in granular and cornified layer, but weak or traceable in the germinative layer and in connective tissue. Of the glycosidase, β-galactosidase was the most reactive and β-glucosidase the weakest. On experimental carcinogenetic process, similar findings were observed.

Through the whole process, PAS reactive polysaccharides were never noticeable. According to results described in this experiment, cancer cells and epithelium undergoing carcinogenesis were not different from the normal matured epithelium in histochemical localization and distribution of enzymes. Therefore, it was supposed that the enzymatic pattern, localization and distribution in epidermal cancer cells were mostly similar to those of normal matured epithelium, and that enzymatic activity was variable in every conditions of epithelium.

Summary

The enzymatic localization and distribution in the skin undergoing carcinogenesis induced by 20-methylcholanthrene were histochemically observed.

1) In the localization and distribution of ACDp-ase, esterase, glycosidase, protein bound sulfhydryl and disulfide groups, no essential differences were noticed from those in normal mature epithelium.

2) ALKp-ase activity became demonstrable in the connective tissue adjacent to invading parts as the epithelium began to proliferate invasively, and related to the grade of stromal reaction in strength. But as carcinization was further promoted ALKp-ase diminished or disappeared from the adjacent connective tissue except for in some capillaries.

3) Aminopeptidase was similar to ALKp-ase in the stadium and places of appearance, but this enzyme was abundant in the ground substance of adjacent connective tissue, mast cells, inflammatory cells if any, and basal cell layer. The positive reacted portion for aminopeptidase activity in the basal cell layer always accompanied those in connective tissue.
**References**


**Histochemical Observations of the Rat Salivary Glands**

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As it is well known, the salivary glands secrete various substances as the so-called serous, mucous or mucoid saliva and reveal very complexed histological structures. The morphological structures and secretory functions of the salivary glands and the relationship between them have not yet been