The Effect of Cyclophosphamide on Experimental Salivary Gland Neoplasia

Robert Sheehan and Gerald Shklar

Department of Oral Medicine and Oral Pathology, Harvard School of Dental Medicine, Boston, Massachusetts 02115

SUMMARY

Fifty young adult male and female albino rats were studied for the effect of systemically administered cyclophosphamide upon chemical carcinogenesis of the submandibular gland. In 1 group of animals, pellets of 9,10-dimethyl-1,2-benzanthracene (DMBA) were implanted into the right submandibular glands. A 2nd group of animals received biweekly i.p. injections of cyclophosphamide (Cytoxan) in addition to DMBA implants into the right submandibular glands. Animals were sacrificed at 13 to 17 weeks after DMBA implantation. In the DMBA animals, well-differentiated epidermoid carcinomas developed within epidermoid cysts. In the DMBA-cyclophosphamide animals, the epidermoid carcinomas developed more rapidly and were more anaplastic histologically. In the DMBA-cyclophosphamide animals, fibrosarcomas and adenocarcinomas developed in addition to the epidermoid carcinomas. The augmentation of the salivary gland carcinogenesis was interpreted in terms of a depression of the immune response to tumor development by the cyclophosphamide.

INTRODUCTION

In studies of chemically induced tumors of hamster buccal pouch, it was found that systemic administration of methotrexate augmented the carcinogenic activity of topically applied DMBA1 (13). The induced tumors not only appeared more rapidly than in DMBA control animals, but the carcinomas were more anaplastic, grew to larger size, and invaded more deeply into underlying connective tissue. Azathioprine was shown to have a similar effect on hamster buccal pouch carcinogenesis, but azathioprine presented a biphasic effect in that it was effective in causing the carcinomas to undergo regression once they had developed (10). The tumors in the azathioprine animals developed more rapidly and were more anaplastic, but they almost completely disappeared after 16 weeks of DMBA application and antimetabolite administration.

It was decided to investigate other anticancer agents and to use another tumor system, the chemically induced rat carcinoma of submandibular gland. The rat salivary gland carcinoma has been studied extensively in this laboratory (3, 4, 12). The technique is well established and standardized (15). A pellet of pure DMBA is implanted in the submandibular gland and a sequence of pathological alterations results in the development of epidermoid carcinomas arising in cyst walls. Tumors other than epidermoid carcinomas occur rarely in the rat (5, 17).

Cyclophosphamide was selected as the anticancer drug for study in this investigation. It has been widely utilized for its chemotherapeutic properties and is effective against a wide variety of neoplasms (6, 9, 14).

Cyclophosphamide can be described chemically as \(N,N\)-bis(\(\beta\)-chloroethyl)-\(N^1, O\)-propylene phosphoric acid ester diamide monohydrate. The mechanism of action of cyclophosphamide and the manner in which it differs from other compounds in the nitrogen mustard class has not been adequately determined (2, 3).

MATERIALS AND METHODS

Fifty young adult male and female albino rats weighing approximately 250 g were separated into 4 groups as follows: Group I consisted of 15 animals implanted with pellets of DMBA; Group II contained 15 animals which received, in addition to the DMBA pellets, biweekly injections of cyclophosphamide; Group III consisted of 10 animals which received biweekly injections of cyclophosphamide; Group IV contained 10 animals which served as the untreated controls.

Animals in Groups I and II were anesthetized with ether, and their right submaxillary gland was surgically exposed. Pellets of DMBA weighing approximately 5 mg were introduced into the glands according to the technique described by Cataldo and Shklar (3). In addition to the pellets of DMBA, animals in Group II, as well as those of Group III, received biweekly injections of cyclophosphamide, 6 mg/kg of body weight, i.p. The crystalline cyclophosphamide was obtained in 50-mg vials and activated with 0.9% NaCl solution immediately prior to injection. The injections were initiated at the time of implantation of the DMBA pellets and continued for the duration of the study. Blood samples were obtained from animals receiving cyclophosphamide, as well as from DMBA and untreated control animals, at the end of 2 weeks and again at the end of 8 weeks. These samples were subjected to hematological evaluation in order to ascertain the effectiveness of the immunosuppressive cyclophosphamide (Table 1).
Animals were maintained on a Purina chow and water diet ad libitum. Three animals from Groups I and II and 2 animals each from Groups III and IV were sacrificed with ether at Weeks 13 to 17. Salivary glands, as well as cervical nodes and major organs, were removed at autopsy. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 4 to 5 μ and stained with hematoxylin and eosin.

**OBSERVATIONS**

**Gross Findings**

The animals in Groups II and III lost some hair and developed patchy areas of alopecia, although they remained healthy otherwise. At the termination of the experiment, the mean weights of these 2 groups were slightly less than the mean weights of either Groups I or IV (Table 2). At 13 weeks following implantation of DMBA pellets, small gross lesions were palpable in the right submandibular gland area. The masses did not differ substantially in size between Group I and Group II animals but were harder and more firm to palpation in Group II animals.

**Microscopic Findings**

**Group I.** Epidermoid cysts had developed by the 13th week and areas of dysplasia, dyskeratosis, and early epidermoid carcinoma were observed within the stratified squamous epithelium of the cyst wall. At Weeks 14 and 15, there were well-established epidermoid carcinomas developing from the epithelium of the cyst wall, and these tumors were of the well-differentiated variety, with the development of large amounts of keratin (Figs. 1 and 2). The tumors tended to be of a papillary configuration, extending into the cyst area and gradually filling it. Penetration of adjacent glandular tissue and connective tissue was observed but was not extensive. There was no spread to regional lymph nodes, and major organs were uninvolved by tumor. All animals in this group had developed epidermoid carcinomas, with the size and degree of invasion increasing from Week 13 to Week 17.

**Group II.** Epidermoid cysts had developed by the 13th week and epidermoid carcinomas had developed from the stratified squamous epithelium of the cyst wall. Well-established carcinomas were apparent, as well as numerous areas of dysplasia. The carcinomas had invaded into the underlying connective tissue and glandular tissue, as well as proliferating into the cystic cavity in papillary configurations. In general, the carcinomas were less well differentiated than those in the Group I animals. Less keratin was developed, a greater degree of cellular pleomorphism was noted (Figs. 3 and 4), a larger number of mitoses and occasional bizarre mitoses were in evidence, and the tumors were larger than those in Group I animals at Weeks 14 and 15.

In addition to the development of epidermoid carcinomas that were larger and less well differentiated than those in the Group I animals, there was the development of fibrosarcomas in 3 animals of Group II and the development of adenocarcinomas in 2 animals (Table 3). In 1 animal the fibrosarcoma, adenocarcinoma, and epidermoid carcinoma appeared to develop simultaneously and were seen to be adjacent to each other and within the parenchymal tissue of the submandibular gland (Figs. 5 to 8).

Both Group II and III animals demonstrated degenerative alterations in lymph nodes, characterized by a decreased density of lymphocytes. Several animals in these groups also demonstrated inflammatory disease in the lungs, characterized by an infiltration of histiocytes and polymorphonuclear leukocytes. One animal in Group II and 1 animal in Group III died before the termination of the experiment. Pneumonia was interpreted as the cause of death in both cases.

**DISCUSSION**

Cyclophosphamide was found to modify the process of chemical carcinogenesis of rat submandibular gland in several ways. The epidermoid carcinomas developed within cyst walls, as in the DMBA-implanted animals, but they developed more rapidly, grew to larger size, and invaded more deeply into the surrounding connective tissue and glandular tissue. The epidermoid carcinomas in the cyclophosphamide-treated animals were less well differentiated, as evidenced by less keratin development and greater pleomorphism. These findings are consistent with the reported effects of other antimetabolite drugs on carcinogenesis, such as the effect of fluorouracil on the rat submandibular gland system (16) and the effects of methotrexate and azathioprine on the hamster buccal pouch system (10, 13). However, cyclophosphamide did not result in eventual regression of the tumors, as buccal pouch tumors in hamsters receiving azathioprine regressed (10). Cortisone was found to augment the development of...
both rat submandibular gland carcinomas (1) and hamster buccal pouch carcinomas (11) but the lesions were not of the more anaplastic variety, as seen in this experiment. An extremely interesting phenomenon was the development of fibrosarcomas and adenocarcinomas in several of the DMBA-implanted and cyclophosphamide-treated animals. Fibrosarcomas are commonly developed as submandibular tumors in DMBA-implanted hamsters but are extremely unusual in rats (3, 5). Adenocarcinomas are occasionally seen in rat salivary glands implanted with carcinogen, but they appear to develop instead of epidermoid carcinomas, rather than together with them.

It would seem that cyclophosphamide acts to depress the immune response, so that tumors not only develop more rapidly but unusual types of tumors also develop together with the more common types. In the DMBA control group, only epidermoid carcinomas developed. In the DMBA-cyclophosphamide experimental group, fibrosarcomas and adenocarcinomas developed in addition to the epidermoid carcinomas. The postulation of a depressed immune response is supported by lymphocyte depression in the cyclophosphamide animals and the development of pulmonary infections in several animals.

The development of more anaplastic tumors has been demonstrated in the buccal tumor system with the use of injections of antilymphocyte serum (8). The results in this study are somewhat similar, but the difference in this tumor system probably accounts for the unusual action in stimulating the development of several varieties of malignant tumor.

REFERENCES

Fig. 1. Well-differentiated epidermoid carcinoma arising in cyst wall within the submandibular gland. H & E, X 100.

Fig. 2. High-power view of well-differentiated epidermoid carcinoma, demonstrating keratin formation and clear demarcation of stratum germinatum. H & E, X 250.

Fig. 3. Moderately anaplastic epidermoid carcinoma arising in submandibular gland. Right, glandular tissue. H & E, X 100.

Fig. 4. High-power view of moderately anaplastic epidermoid carcinoma. Development of keratin is not evident. H & E, X 250.

Fig. 5. Low-power view of tumor arising in submandibular gland. Three patterns are evident, epidermoid carcinoma (top), adenocarcinoma (bottom left), and fibrocarcinoma (bottom right). H & E, X 80.

Fig. 6. High-power view of adenocarcinoma in Fig. 5, demonstrating glandular structures with hyperchromatic nuclei. H & E, X 300.

Fig. 7. High power view of epidermoid carcinoma in Fig. 5. Little keratin is noted and nuclear pleomorphism is evident. H & E, X 300.

Fig. 8. High-power view of fibrosarcoma in Fig. 5. Nuclear hyperchromatism is evident and mitoses are noted. H & E, X 250.
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