DISCOVERY!

Irwin D. Mandel, Editor

Development of Experimental Oral Carcinogenesis and its Impact on Current Oral Cancer Research

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Introduction

I have recently been made aware of my "survivor" status by being awarded a plaque by the Massachusetts Dental Society recognizing me "for fifty years of distinguished service to the profession of dentistry". During these fifty years, I have had a very interesting and rewarding career, acting as a clinician in periodontology, oral pathology, and oral medicine; building and directing departments of oral medicine and oral pathology in two major dental schools (Tufts and Harvard); directing graduate programs in oral pathology and research training programs in oral biology; and developing research laboratories for oral carcinogenesis research in both institutions. I have been extremely fortunate in having a number of exceptional students and associates who joined my research group and collaborated in many of the projects and publications. They have gone on to take academic positions in many dental schools across the country and abroad, and have developed national and international reputations in oral cancer research. Among these are Louis Abbey, Edmund Cataldo, Ellen Eisenberg, George Gallagher, John Giunta, John Jandinski, Norman Meng, Diana Messadi, Onatolu Odukoya, No-Hee Park, Peter Polverini, Helen Santis, Joel Schwartz, Dennis Solt, Stephen Sonis, David Wong, and many others. Teachers may take pride in their research contributions and their publications. However, the immortality of their professional life comes from the success of their students, who will continue the honorable traditions that they have acquired in teaching and in conducting research.

Embarking on a career in dentistry

I was born and grew up in Montreal, Canada. My training and early development were somewhat varied. I received a BS in biology and a DDS from McGill University. During these college days, I spent many summer months as a research technician at a hospital in Montreal, working for a very distinguished German bacteriologist, Herbert Lubinski, who was forced to emigrate to Canada by the Nazi university establishment. Under his guidance, I learned about the elations and frustrations of biological research. The Obs-Gyn department became convinced of the validity of Papanicolau's initial reports on the cytologic diagnosis of cancer and had me trained in this new field. I became one of the first cytology technicians. I had no doubts at that time that I could diagnose early cervical cancer by looking at stained cells. Only later did we find out that other problems, including early pregnancy, could present abnormal-looking cells on the cytologic smears.

I had considered a career in genetics and biological research but decided to go for the financial security of a profession. At that time, there were no research grants on which to survive. My father had been a dentist but died before my college years. Fortunately, in those days, the annual college tuition was only two hundred dollars, and this could easily be earned in summer and part-time jobs. After dental school, I practiced general dentistry for a year and found that periodontal disease (called pyorrhea) was a major problem in my practice. I attended a lecture by Irving Glickman and was very impressed with his knowledge and treatment of periodontitis. I met him after the lecture and asked him about the possibility of graduate training in Boston. He said "spend six months with me and you'll know everything there is to know about periodontology—spend a year with me and you'll become an authority". So I became an "authority", got my periodontal board certification, and then spent some ten years treating periodontitis and doing periodontal research with Glickman in Boston, studying osteoporosis and some of its endocrine relationships. I had also studied pathology with Edward MacMahon and oral medicine with Francis McCarthy, two outstanding teachers and clinicians, and gradually became more interested in oral mucous membrane diseases and oral cancer. I also met Irving Meyer, a gifted oral surgeon, who taught me a great deal about oral cancer and its management. We eventually collaborated in many projects dealing with oral cancer. The National Cancer Institute also started funding training programs in oral cancer at this time, and I directed this program at Tufts and later at Harvard. Mortality from oral cancer used to be well over 50%, and successful surgical and/or radiation therapy usually resulted in severe facial deformity. It was obvious that new approaches were needed and would require new concepts based on basic biological research.

The turn to cancer research

My curiosity about the fundamental nature of cancer stimulated me to undertake research in the cancer field, and for the past forty years I have been carrying out research on experimental carcinogenesis, using a number of animal models. Our research has given us new insight into the basic biology of cancer

development, including molecular genetic mechanisms, and immunologic controls. It has also helped to answer a number of fundamental questions related to cancer management. For example, the old concept that biopsy was dangerous and could spread cancer could be tested by producing cancers in animals, and then subjecting them to incision and trauma. This did not spread the cancer in any way (Shklar, 1968). These results were appreciated by my pathologist friend Shields Warren, who always doubted the old concept and advised early biopsy.

Most of my research on carcinogenesis was carried out in an experimental cancer model using the hamster buccal pouch and topical application of a chemical carcinogen. This model was first reported by John Salley in 1954 and has turned out to be the optimal model for the study of oral cancer (Shklar, 1972; Gimenez-Conti and Slaga, 1993). Initially, we applied our clinical experience to test the validity of the model. Chronic irritation in the mouth was considered to be a risk factor in oral cancer development, and we found that croton oil, a potent mucosal irritant, enhanced experimental oral carcinogenesis when applied in conjunction with the carcinogen 7,12-dimethylbenz(a)anthracene, otherwise known as DMBA (Silberman and Shklar, 1963). Extensive further studies have shown the hamster buccal pouch model to be the ideal model not only for oral cancer research, but also one of the better models for the study of cancer generally. The squamous cell carcinomas were found to develop slowly from an initial pre-cancerous lesion similar to human dysplastic leukoplakia (Santis *et al.*, 1964). The malignancies gradually became invasive and had the potential of metastasis to regional (cervical) lymph nodes. The tumors resembled their human counterparts both grossly and microscopically (Malament and Shklar, 1981), and were found to have similar metabolic markers (Shklar, 1965; Solt, 1981; Solt and Shklar, 1982; Shin *et al.*, 1990) and oncogene expression (Wong, 1987; Husain *et al.*, 1989; Wong *et al.*, 1989). Initiation and promotion of the tumors could be demonstrated in this model (Odukoya and Shklar, 1982, 1984). An anaplastic carcinoma model for the hamster buccal pouch was developed for the study of metastatic spread (Meng *et al.*, 1982). Squamous cell carcinoma could also be induced in hamster buccal pouch cells in culture, and this also resulted in anaplastic carcinoma when transplanted into the buccal pouches of syngeneic hamsters (Schwartz and Shklar, 1997). A cell line was developed by Odukoya *et al.* (1983) from a hamster buccal pouch carcinoma, and this cell line (HCPC-1) has been widely used in cancer research.

Immunological studies

Our research on oral carcinogenesis has served to elucidate many aspects of cancer biology and to illustrate some of the major risk factors related to human oral cancer. Studies on the hamster pouch tumors were among the first to illustrate the role of the immune system in the control of tumor development and to offer experimental evidence for the concept of immunosurveillance, first outlined by Paul Ehrlich. Our major aim was to find agents that could inhibit or prevent the development of the hamster pouch carcinomas. Sydney Farber, the father of chemotherapy, had shown that antifolate drugs could act against childhood leukemia. He suggested that I use methotrexate to inhibit oral cancer development in my experimental animals and gave me some of the drug. Use of the methotrexate resulted in faster tumor growth and deeper invasion rather than inhibiting carcinogenesis. Neither my associates nor I could understand this result until we realized that methotrexate was a potent immunosuppressive agent. Immunosuppressive agents such as methotrexate (Shklar *et al.*, 1966), cortisone (Shklar, 1966), and specific anti-lymphocyte serum (Woods, 1969) were found to enhance experimental oral carcinogenesis, while immuno-enhancing agents such as BCG (Giunta *et al.*, 1974) and levamisole (Eisenberg and Shklar, 1977) were found to inhibit experimental oral carcinogenesis. This research on the immune response was particularly satisfying to me, since some influential cancer researchers had written, earlier, that the hamster pouch was an immunologically privileged or protected site, and that human tumors could be transplanted to this site. I wasted almost a year trying to transplant human tumors to the hamster pouch and had no success. It finally occurred to me that not all cancer research was carefully or honorably performed, and I now tell my students that it's always a good idea to repeat some published work if they have doubts about the results. It may have been misguided or even fraudulent. Following up our immune studies, we also found that peritoneal macrophages and other immune effectors demonstrated a decrease in Fc and C_3 receptors in the tumor-bearing animals, indicating a diminished tumor-killing capacity through antibody-dependent cellular cytotoxicity (Antoniades *et al.*, 1984). A decreased density of Langerhans cells and loss of their complex dendritic networks were also found in the buccal pouches of carcinogen-treated hamsters (Schwartz *et al.*, 1985). Mast cells were found gradually to infiltrate and degranulate during experimental carcinogenesis, releasing their cytokines (Flynn *et al.*, 1991).

We also found that the major known risk factors in oral cancer, such as alcohol and tobacco, could be studied by experimental pathology, which offered a clearer picture of the mechanism than that obtained from epidemiologic studies. Alcohol was found to enhance experimental oral carcinogenesis, and liver damage could be correlated with the effect of alcohol ingestion (Freedman and Shklar, 1978). Recently, Altuwairgi *et al.* (1995) were able to show that N-nitrosonornicotine, one of the major carcinogenic agents in tobacco, was able to act as a promoter in producing experimental hamster oral cancers with subcarcinogenic doses of DMBA. Herpesvirus infection was also found to promote or enhance experimental oral carcinogenesis induced by simulated snuff dipping (Park *et al.*, 1986).

Molecular biology and molecular genetics

These studies in the experimental pathology of oral cancer in the hamster buccal pouch model have led to a variety of studies using the disciplines of molecular biology and molecular genetics. Those studies, in turn, have led to molecular studies in the development of human oral cancer (Wong *et al.*, 1996). In other cases, molecular studies applied to human cancer have stimulated further animal research. David Wong has made major discoveries in the molecular biology of oral cancer. Oncogenes expressed in hamster pouch carcinomas were found to include C-erb-B (Wong, 1987), Ha-ras (Husain *et al.*, 1989; Kwong *et al.*, 1992), Ki-Ras (Wong *et al.*, 1989), and mutant p53 (Schwartz *et al.*, 1993). Current concepts of neoplastic transformation suggest that there must be a critical mass of genetic injury to the cell, and that this may require a multi-step accumulation of oncogene activity. A new

putative oral cancer suppressor gene (doc-1) was discovered by Todd *et al.* (1995) in a study of hamster buccal pouch carcinogenesis, using the technique of subtractive hybridization. A human counterpart of this gene was found and was cloned and mapped by Tsuji *et al.* (1996).

The role of growth factors in carcinogenesis was also studied. Wong and associates (1988) demonstrated the important role of TGFα in experimental oral cancer development, and Shin and associates (1990) studied the expression of epidermal growth factor receptor in hamster buccal pouch carcinogenesis.

Nutritional studies

By 1980, our laboratory became successful in demonstrating the inhibition and prevention of experimental oral cancer with the use of anti-oxidant micronutrients and other agents. Following the demonstration by Bollag (1972), that retinoic acid could inhibit the development of experimental skin carcinomas, we demonstrated that systemically administered 13-cis-retinoic acid could inhibit carcinogenesis of the hamster buccal pouch and hamster tongue (Shklar *et al.*, 1980a,b). However, there were problems with retinoids as experimental cancer-preventive agents. In high doses, they presented significant toxicity to the liver and kidneys. Furthermore, some studies had shown an exacerbating effect of retinoids on hamster buccal pouch carcinogenesis, rather than an inhibition (Levij and Polliack, 1968; Shklar and Schwartz, 1988), and others found no significant effect on carcinogenesis (Gilmore and Giunta, 1981). Alpha-tocopherol and the carotenoids, beta carotene and canthaxanthin, were found to exert a greater and more consistent inhibition of experimental hamster buccal pouch carcinogenesis, without appreciable toxicity. Alpha-tocopherol (vitamin E), administered systemically by mouth, could inhibit oral carcinogenesis (Shklar, 1982) and could completely *prevent* tumor development if the hamster tumor model was altered so that the amount of carcinogen application was reduced. Tumor induction time was increased from 12 weeks to 29 weeks as a result. However, 100% of control animals had carcinomas, and none of the DMBA-vitamin E animals had tumors at the termination of the experiment (Trickler and Shklar, 1987). Vitamin E was also found to inhibit hamster buccal pouch carcinogenesis when applied topically on days alternating with painting with carcinogen (Odukoya *et al.*, 1984). We also demonstrated that vitamin E could cause established squamous cell carcinomas of hamster buccal pouch to *regress*, if the nutrient were injected into the tumor or close to the tumor site (Shklar *et al.*, 1987).

Along with the investigations of vitamin E on hamster buccal pouch carcinogenesis, studies were carried out with beta carotene as an anti-cancer agent. This micronutrient is relatively non-toxic and was shown to be an effective inhibitor of experimental oral carcinogenesis. Tumor development was inhibited by topical application (Suda *et al.*, 1986, 1987) or systemically by oral administration (Schwartz *et al.*, 1989). Regression of established squamous cell carcinomas could be caused by local injection (Schwartz and Shklar, 1987, 1988). Canthaxanthin, a carotenoid that does not convert to retinoid, was also found to be an effective anti-cancer agent in this experimental system, whereas 13-cis retinoic acid, injected into the tumor site, did not result in tumor regression (Schwartz and Shklar, 1988). These experiments suggested that beta-carotene remained effective as an anti-cancer agent when used topically or injected, because it did not convert to retinoid in appreciable amounts.

The finding that an algae extract, containing alpha-tocopherol and a number of carotenoids, was a highly effective anti-cancer agent in this experimental system (Schwartz and Shklar, 1987) suggested that there could be a synergistic effect between beta-carotene and alpha-tocopherol. This indeed proved to be the case: The mixture could cause regression of established carcinomas of the buccal pouch even when given orally (Shklar *et al.*, 1989), while the individual anti-oxidant nutrients, administered orally, caused no tumor regression. A further mixture—containing beta-carotene, alpha-tocopherol, reduced glutathione, and ascorbic acid—was even more effective in tumor regression (Shklar *et al.*, 1993). Glutathione was shown to inhibit experimental oral carcinogenesis (Trickler *et al.*, 1993; Schwartz and Shklar, 1996), but vitamin C had no such effect (Shklar and Schwartz, 1996a). Ascorbic acid stabilizes the activity of vitamin E, thereby making it more effective.

The hamster buccal pouch model has been used to demonstrate the anti-cancer activity of a number of food extracts, including onion (Niukian *et al.*, 1987), garlic (Meng and Shyu, 1990), and a protease inhibitor from soybeans (Messadi *et al.*, 1986). Cancer inhibition in the cheek pouch model was also demonstrated by cyclo-oxygenase inhibitors such as aspirin, indomethacin, and ibuprofen (Perkins and Shklar, 1982; Cornwall *et al.*, 1983).

The animal studies in oral cancer inhibition by micronutrients have served as an experimental basis (Shklar and Schwartz, 1993) for a number of clinical trials with micronutrients as cancer-chemopreventive agents. Benner and associates (1993) found that vitamin E could effect regression of oral leukoplakia. Recently, Heinonen and associates (1998) were able to show that even a small daily dose of vitamin E could reduce the incidence of prostate cancer by 30%. Human trials of beta-carotene have also been carried out, demonstrating a response of oral leukoplakia (Garewal *et al.*, 1990), with less toxicity than retinoids, but human trials on cancer prevention have proved to be disappointing (Heinonen *et al.*, 1998).

Investigating mechanisms of cancer inhibition

Once the notable anti-cancer activity of micronutrients was demonstrated in the excellent hamster model, further investigations were necessary to develop concepts of the mechanism of action. These agents were shown to act in four major ways: (1) They are potent anti-oxidants and are capable of trapping peroxyl free radicals; (2) they are immuno-enhancing agents; (3) they stimulate the activity of tumor suppressor genes such as p53, and dysregulate the activity of various oncogenes; and (4) they inhibit tumor angiogenesis.

Immuno-enhancement has been well-demonstrated by the maintenance of the immune function of Langerhans cells near the tumor site (Schwartz *et al.*, 1985), by stimulating macrophages to bring tumor necrosis factor to the tumor site (Shklar and Schwartz, 1988), and by stimulating cytotoxic lymphocytes (Schwartz *et al.*, 1990). In fact, these immune studies (Shklar *et al.*, 1990) were among the first to offer experimental evidence for the classic concept of "immunosurveillance", originally offered by Ehrlich. The nutrients appeared to act through a common pathway (Shklar

and Schwartz, 1994) through which they stimulated the induction of heat-shock proteins (hsp 70, 90) (Schwartz *et al.*, 1990), which, in turn, stimulated the expression of the p53 tumor suppressor protein (Schwartz *et al.*, 1993). The nutrients were also shown to inhibit tumor angiogenesis, by their inhibition of angiogenesis-inducing agents such as TGFα (Shklar and Schwartz, 1996b). Tumor angiogenesis has become an important concept of tumor development, whereby the blocking of the tumor blood supply by anti-angiogenesis agents would cause the tumor to atrophy and shrink (Folkman, 1990). Vitamin E and beta-carotene were also shown to destroy a variety of human cancer cells in culture by direct action on cell metabolism (Schwartz and Shklar, 1992).

Future directions

Animal models for disease, such as the hamster buccal pouch, continue to be important for the development of our knowledge of etiology, pathogenesis, and therapy. An understanding of the complex etiology of cancer, at the molecular level, will result in better concepts of how to prevent it. The ultimate aim of medicine must be disease *prevention* rather than disease treatment. Animal experimentation has helped to direct us to the mechanisms of cancer prevention. Future research requires extensive knowledge of molecular genetics, protein biochemistry, immunochemistry, and other new experimental disciplines. My students represent the generation that will solve many of these problems. I am able to appreciate their molecular research only because I have the good fortune of being married to a biochemist-immunologist, Se-Kyung Oh, who has the patience to explain the current research to me in simple terms that I can understand!

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