

Inhibition of Branching Morphogenesis of Mouse Fetal Submandibular Gland by Sodium Fluoride – Protection by Epidermal Growth Factor

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Abstract. As an initial step to study the effect of sodium fluoride on the oral environment, we investigated how sodium fluoride (NaF) affects the branching morphogenesis of fetal mouse submandibular gland (SMG). When mouse SMG was prepared from the embryo at 13 days post prenatal stage and cultured, gradual development of branching morphogenesis was observed. Addition of NaF affected this morphological change in bimodal fashions. At a lower concentration of NaF (<2 μ M), the branching morphogenesis was slightly enhanced, whereas at a higher concentration of NaF (4~8 μ M), it was almost completely inhibited. The inhibitory effect of NaF at the higher concentration was abrogated by simultaneous addition of epidermal growth factor (EGF), but not by 5 α -dihydrotestosterone (DHT) or insulin-like growth factor (IGF). These data demonstrate that EGF can effectively reduce the cytotoxic activity of NaF at micromolar concentration.

Since 1945, when the fluoridation of the water supply was introduced, fluoride has been used not only as an additive to drinking water, but also as a mouthwash, toothpaste, and cement for dental use such as glass ionomer cement, due to its inhibitory effect on dental caries and its stimulatory effect on remineralization (1). Considering its safety, however, some researchers oppose the utilization of fluoride to prevent dental caries. Some kinds of disorders caused by excess amounts of fluoride, such as speckled tooth and early

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skeletal fluorosis, have been reported. In this context, the cytotoxicity of fluoride has been well investigated (2-7). However, the study of its effect on the developing organ has been limited (8,9). The aim of the present study was to investigate the effect of sodium fluoride (NaF) on fetal mouse submandibular gland (SMG), a well-established organ development model. Branching morphogenesis of the mouse SMG is dependent on cell-cell interaction between and within epithelium and mesenchyme. Such an interaction is also seen in other organs including lung, kidney and pancreas. It is known that branching morphogenesis *in vitro* is stimulated by addition of epidermal growth factor (EGF) (10-12), insulin-like growth factor (IGF) (11) and 5 α -dihydrotestosterone (DHT) (13-17). Therefore, we also tested the possibility that any of these growth factors and hormone may modify the effect of NaF on SMG.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: BGJb medium (Gibco BRL, NY, USA); sodium fluoride (NaF) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); EGF (Upstate Biotechnology, Lake Placid, USA); IGF (Techne, Minneapolis, USA); DHT (Sigma Chem. Co., Ltd., Minnesota, USA).

Preparation of salivary glands. Pregnant ICR strain mice were purchased from Sankyo Laboratories (Shizuoka, Japan). The submandibular/sublingual gland complex, referred to as SMG, was dissected from E13 stage mouse embryos.

Organ culture. Mouse fetal SMGs were cultured on Transwell clear filters (Corning, NY, USA). The filters were floated on 2 ml serum-free BGJb medium (Gibco BRL). The medium was supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin. The organs were incubated at 37°C in a 5% CO₂ atmosphere for up to 3 days. Branching morphogenesis of the organ culture of SMG were observed and photographed under photomicroscope

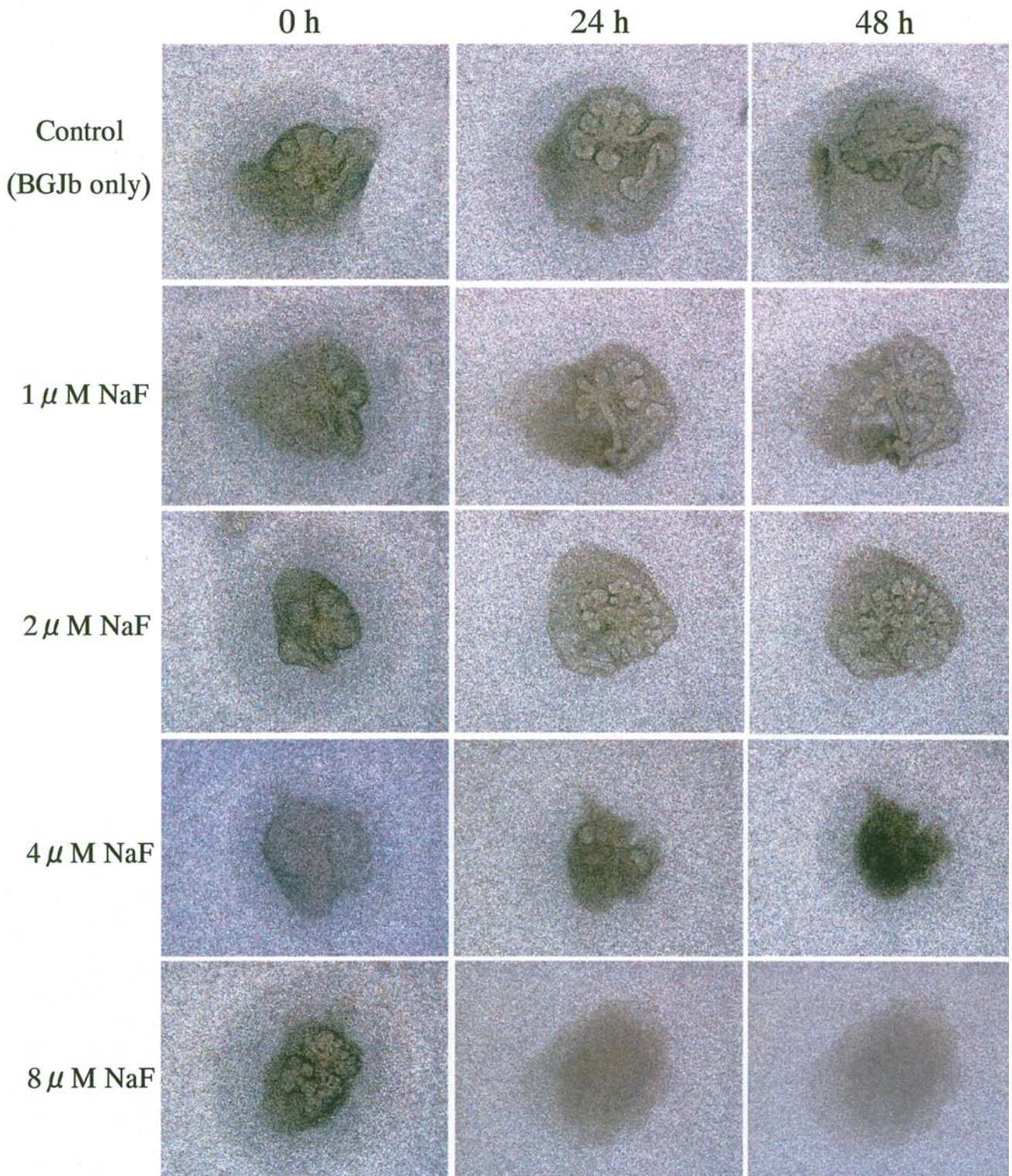


Figure 1. Effect of NaF on the branching morphogenesis of SMG. SMG from E13 mouse embryo was cultured for 0, 24 or 48 hours with the indicated concentrations of NaF.

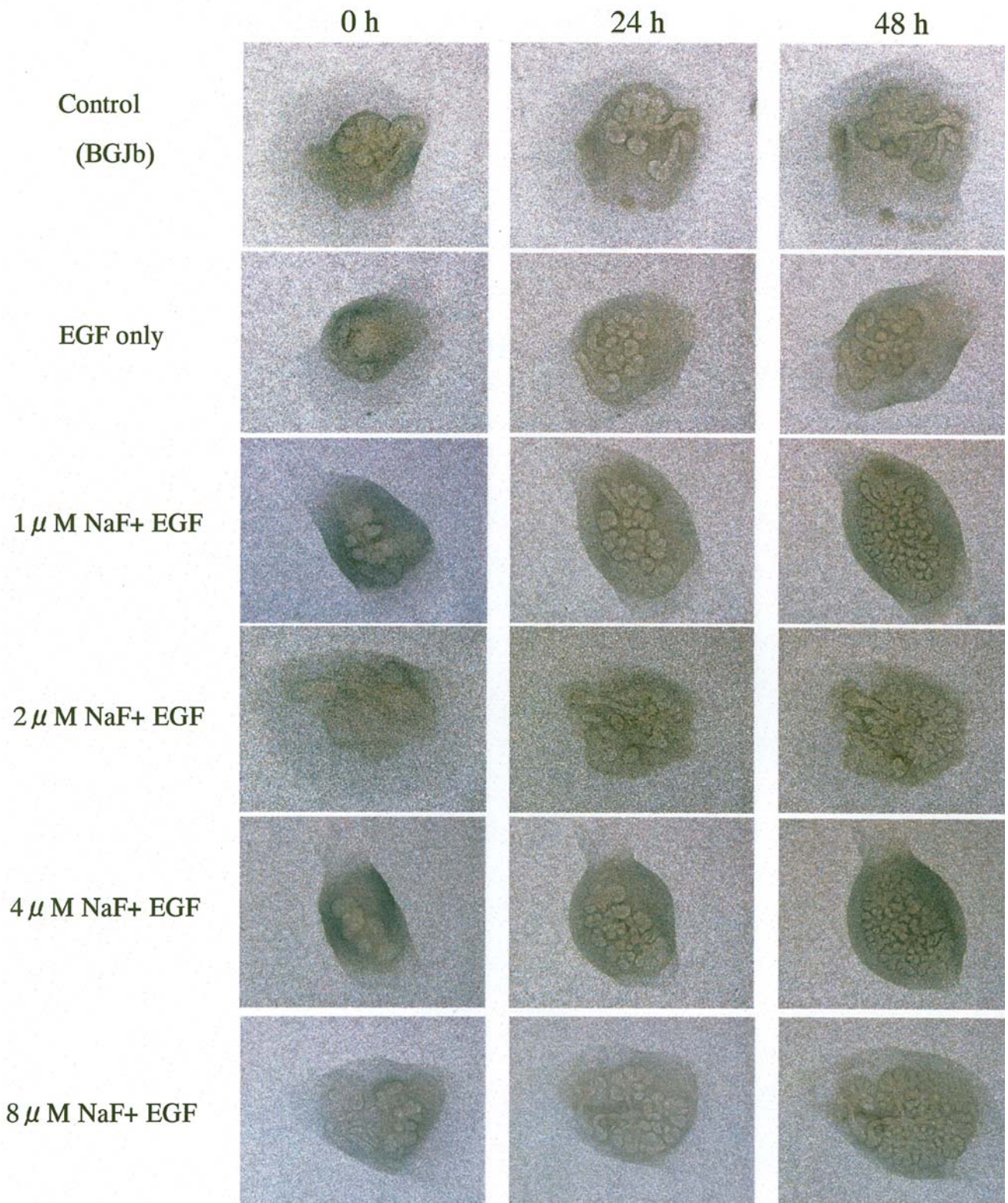


Figure 2. Combination effect of NaF and EGF on the branching morphogenesis of SMG. SMG from E13 mouse embryo was cultured for 0, 24 or 48 hours with the indicated concentrations of NaF in the presence of 2 ng/ml EGF.

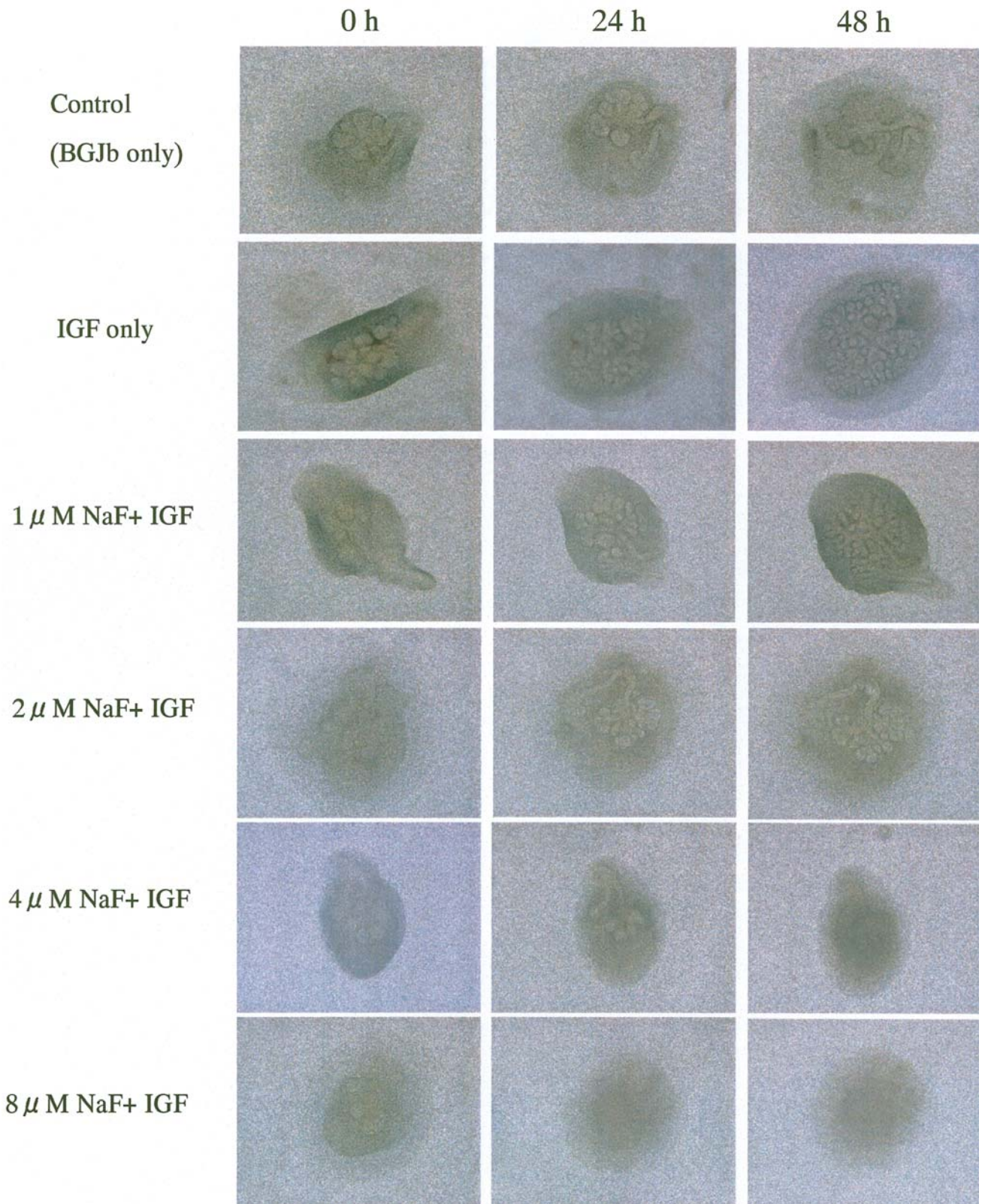


Figure 3. Combination effect of NaF and IGF on the branching morphogenesis of SMG. SMG from E13 mouse embryo was cultured for 0, 24 or 48 hours with the indicated concentrations of NaF in the presence of 1 ng/ml IGF.

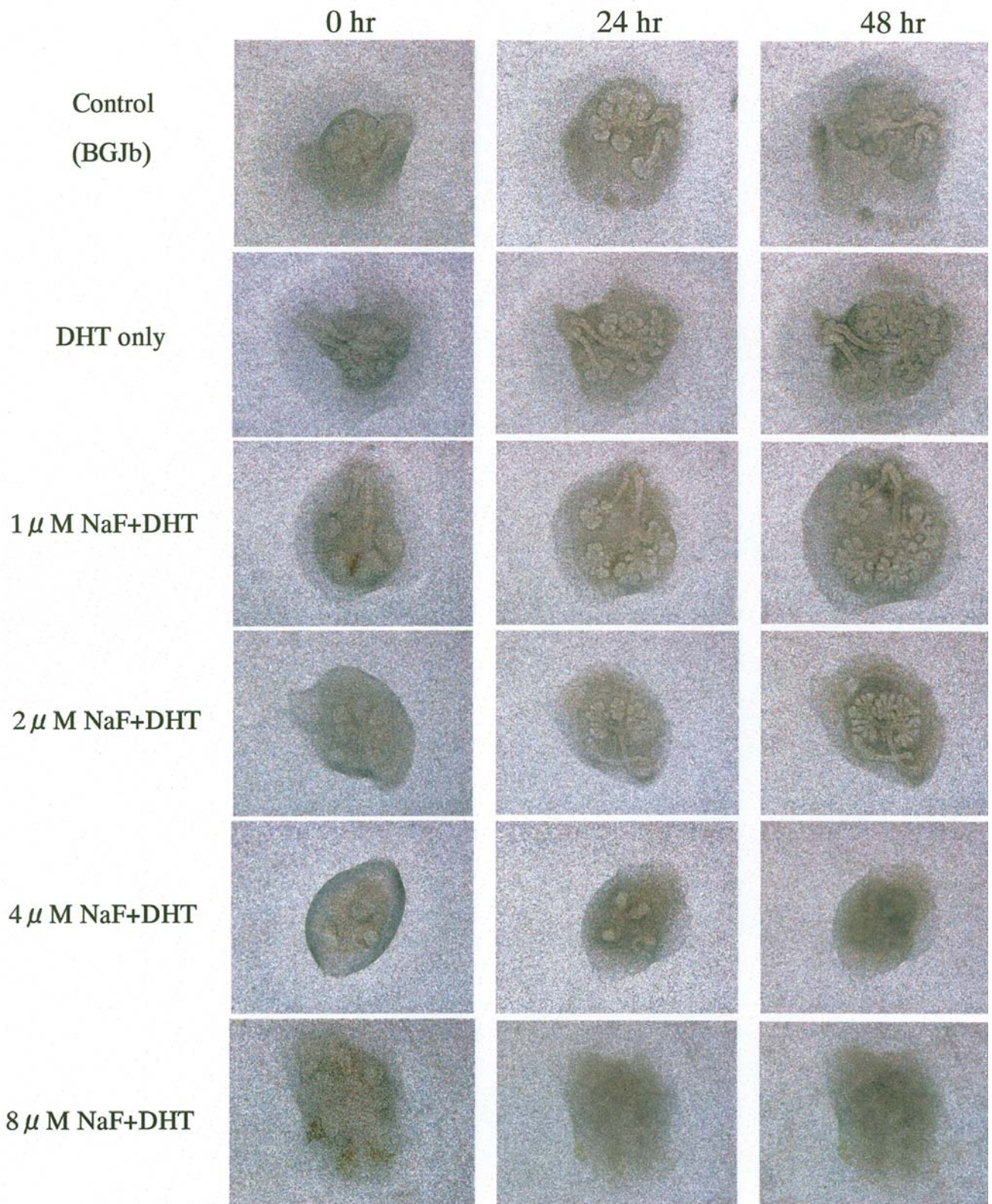


Figure 4. Combination effect of NaF and DHT on the branching morphogenesis of SMG. SMG from E13 mouse embryo was cultured for 0, 24 or 48 hours with the indicated concentrations of NaF in the presence of 10 μ g/ml DHT.

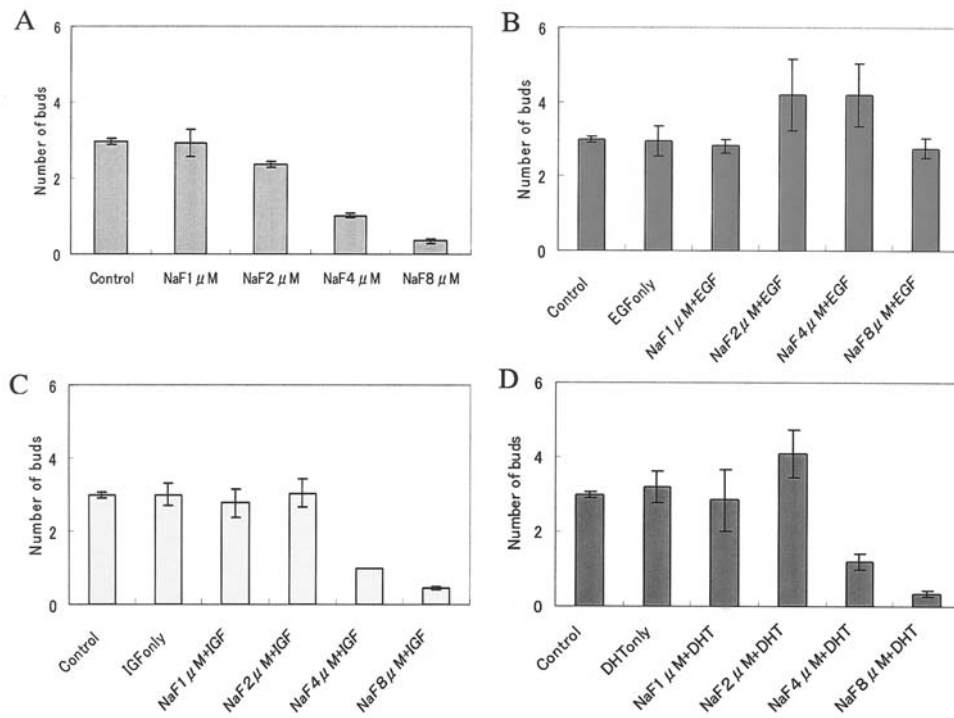


Figure 5. Short-term (24 hours) effects of NaF and growth factors or hormone on the branching morphogenesis of SMG. Data of the first 24 hours in Figures 1, 2 and 3 were summarized. The extent of growth of SMG is expressed as the ratio of number of buds at 24 hours / the number of buds at 0 hour. Each value represents mean \pm S.E. from 7 determinations.

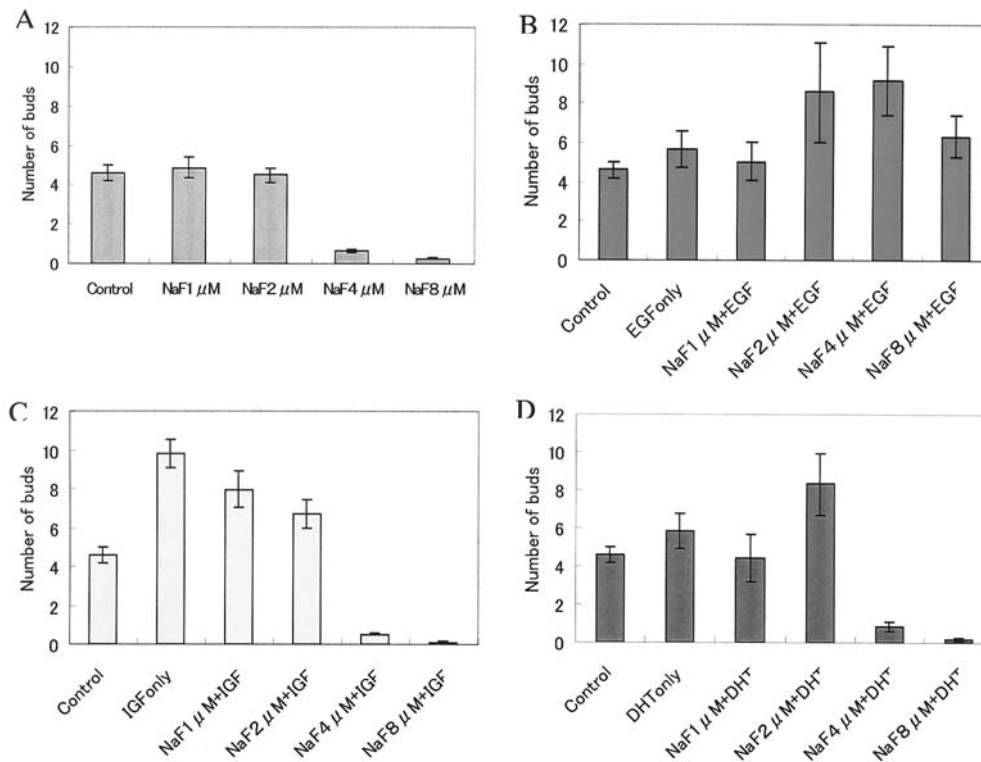


Figure 6. Long-term (48 hours) effects of NaF and growth factors or hormone on the branching morphogenesis of SMG. Data of 48 hours in Figures 1, 2 and 3 were summarized. The extent of growth of SMG is expressed as the ratio of number of buds at 48 hours / the number of buds at 0 hour. Each value represents mean \pm S.E. from 7 determinations.

(Olympus, Tokyo, Japan) at the time of SMG removal, and 24 and 48 hours thereafter, and the number of end buds was counted at each time point. Each experiment was repeated at least three times. Four different NaF concentrations (1, 2, 4, 8 μM) were used for this experiment. In separate culture, epidermal growth factor (EGF)(2 ng/ml), 5 α -dihydrotestosterone (DHT)(10 $\mu\text{g/ml}$) and insulin-like growth factor (IGF)(1 ng/ml) were added to each concentration of NaF solution.

Results

Effect of NaF on the growth of SMG. The number of buds increased 3-fold during the first 24 hours (from 6.86 ± 1.35 (n=7) to 20.3 ± 3.68 (n=7) (Figures 1 and 5A) and 4.5-fold during 48 hours (from 6.86 ± 1.35 (n=7) to 30.9 ± 7.43 (n=7) (Figures 1 and 6A) in the control SMG culture. When NaF was added to the SMG culture, the number of buds changed in a bimodal fashion. The number of buds was slightly increased at lower concentrations of NaF (1 μM), but decreased at higher concentrations (>2 μM) (Figures 5A and 6A). The inhibitory effect of NaF became apparent above 4 μM (Figure 1).

Counteraction of EGF. EGF (2 ng/ml) alone did not significantly affect the growth of SMG, but almost completely inhibited the growth suppressive effect of NaF (4 and 8 μM) (Figures 2, 5B and 6B). The combination treatment of NaF (2 and 4 μM) and EGF resulted in a 41-98% increase in the number of buds.

Counteraction of IGF. IGF (1 ng/ml) alone stimulated the growth of SMG at later stage (48 hours), but did not inhibit the growth suppressive effect of NaF (4 and 8 μM) (Figures 3, 5C and 6C).

Counteraction of DHT. DHT (10 $\mu\text{g/ml}$) alone slightly increased the growth of buds at later stage (48 hours), but did not affect the growth suppressive effects of NaF (4 and 8 μM) (Figures 4, 5D and 6D). However, combination treatment of NaF (2 μM) and DHT resulted in a 38-80% increase in the number of buds.

Discussion

The present study demonstrates, for the first time, that micromolar concentration of NaF suppressed the growth of SMG (CC_{50} =2-4 μM). It has previously been reported that a much higher concentration of NaF is required to kill normal human cells (CC_{50} of NaF against gingival fibroblast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF was 5.5, 9.2 and 5.9 mM, respectively) and human cancer cell lines (CC_{50} of NaF against human oral squamous cell carcinoma HSC-2, HSC-3, melanoma A375, hepatoma HepG2 and promyelocytic leukemia HL-60 was 2.9, 3.2, 2.8,

5.2 and 2.7 mM, respectively)(18). This indicated the sensitivity of SMG against NaF was one thousand-fold higher than that of human normal and tumor cells. This different sensitivity between SMG and human cultures against NaF may be due to the different culture systems. SMG is cultured in serum-free medium, whereas the human cells are cultured in culture medium supplemented with 10% serum, which should contain growth factors. It therefore remains to test the effect of autologous (mouse) and heterologous (human) sera on the growth of SMG.

We found a slight growth promoting action of NaF at a lower concentration (1 μM). This may be the hormesis, a beneficial effect observed in many toxic substances such as cadmium, organophosphorus compounds, endocrine disruptors and even radiation (19). We have recently found antioxidants such as vitamin C and gallic acid showed similar biphasic effects (unpublished data). However, it should be noted that the hormetic effect of NaF (6%) is much smaller than that of others (usually 30-40%) (19).

We found that all growth factors and hormone (EGF, IGF, DHT) stimulated the growth of SMG in the presence of non-cytotoxic concentrations of NaF (1-2 μM), and that the growth suppressive effect of NaF at higher concentrations (4 and 8 μM) was abrogated by the addition of EGF, but not by IGF nor DHT. These data suggest that the two growth factors EGF and IGF may act differently. EGF may inhibit the cytotoxic activity by promoting cell survival (*via* activation of AKT and STAT 3/5) or inducing proliferation (*via* activation of ERK1/2) (20). On the other hand, IGF may augment the beneficial growth-promoting effect of NaF. Further study is required to identify the action mechanisms of NaF and growth hormones.

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