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Modulation of Branching Morphogenesis of Fetal Mouse Submandibular Gland by Sodium Ascorbate and Epigallocatechin Gallate

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Abstract. *As an initial step to study the effect of antioxidants on the oral environment, we here investigated how sodium ascorbate and (-)-epigallocatechin 3-O-gallate (EGCG) affect the branching morphogenesis of the fetal mouse submandibular gland (SMG). When mouse SMG was prepared from the embryo at 13-day post prenatal stage and cultured, gradual development of branching morphogenesis was observed. Addition of sodium ascorbate affected this morphological change in a bimodal fashion. At lower concentrations of sodium ascorbate (0.25 ~ 2.27 mM), the branching morphogenesis was slightly but significantly (about 60%) enhanced, whereas at higher concentrations of sodium ascorbate (6.82 ~ 10.1 mM), the branching morphogenesis was inhibited. The addition of EGCG failed to stimulate, but inhibited the branching morphogenesis in a dose-dependent manner. These data support that the addition of a lower concentration of sodium ascorbate is essential to stimulate the growth of SMG, and that sodium ascorbate, but not all antioxidants, induces hormesis (beneficial action at lower concentration) in the present SMG system.*

Sodium ascorbate (Vitamin C) is involved as a cofactor in many hydroxylation reactions of metabolic pathways (such as proline hydroxylation during collagen formation (1), the hydroxylation of dopamine during catecholamine biosynthesis (2), prolyl hydroxylation of hypoxia inducible factor-1α (HIF-1α) during its oxygen-dependent degradation (3), thus maintaining the normal function and structure of the body. Sodium ascorbate has two opposing properties, antioxidant and pro-oxidant. Sodium ascorbate scavenges active oxygens and radicals (O_2^-, hydroxyl radical, NO, DPPH), but produces its own (ascorbate) radical, oxidizes methionine (an essential amino acid), produces hydrogen peroxide, and stimulates or inhibits the growth of many cultured cells, depending on the concentration (4).

(-)-Epigallocatechin 3-*O*-gallate (EGCG) has unique structural characteristics. EGCG can be classified either as one of the condensed tannins, since it is produced by the condensation of gallic acid (structural unit of tannin), or as a flavoniod, since it has the C_6-C_3-C_6 backbone (5). EGCG is a main constituent of green tea. EGCG also has both antioxidant and oxidant properties. The O_2^- scavenging activity of tannins (2450-4700 SOD unit/ml) exceeded that of vitamin C (360 SOD unit/mg) (6). The NO scavenging activity of EGCG exceeded that of lignins (Satoh *et al.*, unpublished data) and induced some tumor-specific cytotoxicity against cancer cell lines (TS value=4.1) (7). EGCG has inhibited chemically-induced carcinogenesis (8) and induced apoptotic cell death in human prostate cancer (9) and stomach cancer cell lines (10). Recently, EGCG has been shown to inhibit the production of vascular endothelial growth factor (VEGF), which plays a significant role in angiogenesis (11) and the infiltration of cancer cells (12). EGCG reduced the infectiousness of the influenza virus by binding to the spike (glycoprotein) of the virus (13).

The branching morphogenesis of the mouse SMG is dependent on the cell-cell interactions between and within the epithelium and the mesenchyme. Such interactions are also seen in other organs including lung, kidney and pancreas. It is known that the branching morphogenesis *in*

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Key Words: Sodium ascorbate, epigallocatechin gallate, branching morphogenesis, hormesis, SMG.

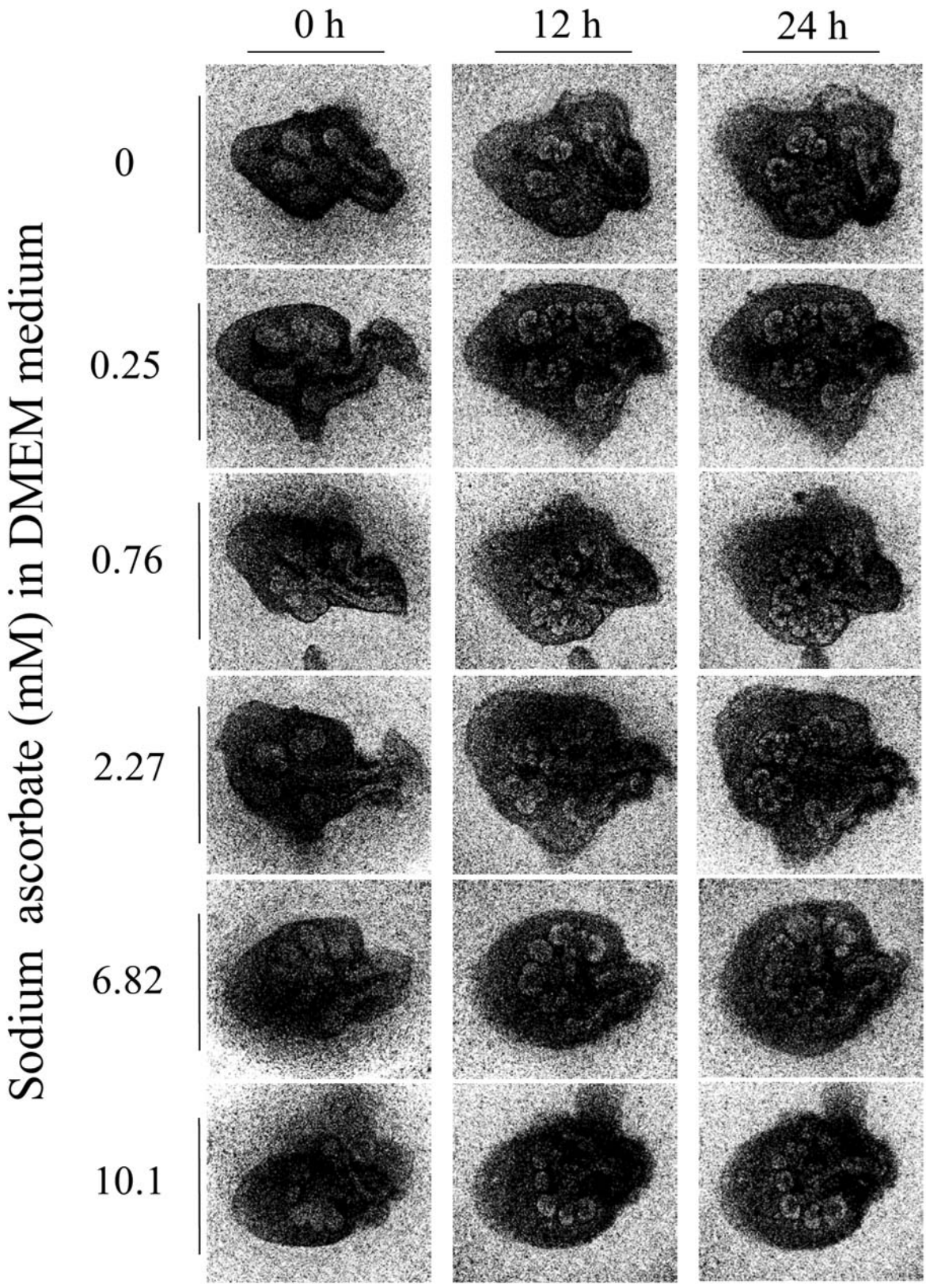

Figure 1. *Morphological changes induced by sodium ascorbate in organ culture of SMG. SMG from E13 mouse embryo was cultured for 0, 12 or 24 hours with the indicated concentrations of sodium ascorbate and photographed under a photomicroscope.*

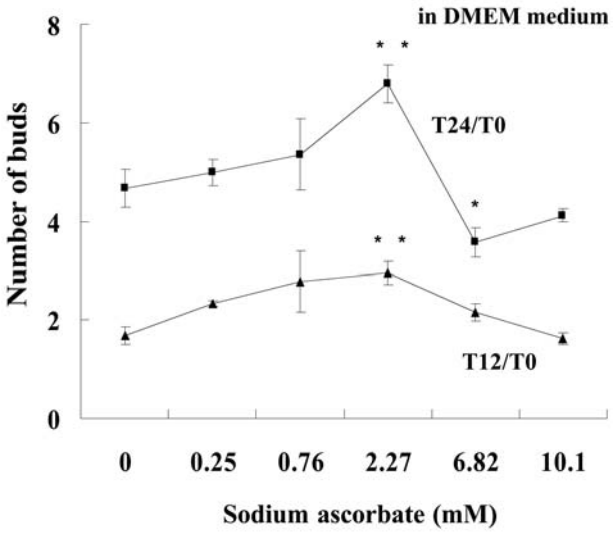

Figure 2. *Dose-effect of sodium ascorbate on the branching morphogenesis of SMG. SMG was cultured as described in Figure 1, and the buds in the SMG were counted after short-term (12 hours) and long-term (24 hours) incubation. The extent of growth of SMG was expressed as the ratio of number of buds at 12 or 24 hours / the number of buds at 0 hour. Each value represents mean±S.E. from 6 determinations. *p<0.05, **p<0.01 vs. control.*

vitro is stimulated by the addition of epidermal growth factor (EGF) (14-16), fibrobrast growth factor (FGF) (17), insulin-like growth factor (IGF) (15), hepatocyte growth factor (HGF) (18), transforming growth factor-α (TGF-α) (15), transforming growth factor-β (TGF-β) (15, 19) and 5α-dehydrotestosterone (DHT) (20-24).

However, no study of the effect of sodium ascorbate and EGCG on the developing organ has been reported. The aim of the present study was to investigate the effects of the antioxidants, sodium ascorbate and EGCG, on fetal mouse SMG, a well-established organ development model.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: BGJb medium, DMEM medium (Gibco BRL, NY, USA); sodium ascorbate (Tokyo Kasei, Tokyo, Japan); EGCG (Wako Pure Chemical Industries, Osaka, Japan).

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 the E13 stage of the mouse embryo.

Organ culture. Mouse fetal SMGs were cultured on Transwell clear filters (Corning, NY, USA). The filters were floated on 2 ml serum-free BGJb or DMEM medium (Gibco BRL). The medium was supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate. The organs were incubated for 0, 12 and 24 hours at 37˚C in a 5% CO_2 atmosphere with various concentrations of sodium ascorbate or EGCG. The branching morphogenesis of the organ culture of SMG was observed and photographed under a photomicroscope (Olympus, Tokyo, Japan). The number of end buds was counted at each time-point. Each experiment was repeated at least three times.

Statistical treatment. Differences between the control and treated groups were evaluated by Student's *t*-test.

Results

Effect of sodium ascorbate on the growth of SMG. When control SMG cultures were incubated in the serum-free DMEM medium, the number of buds in the control SMG culture was increased 1.6-fold during the first 12 hours (from 9.66±0.92 (n=6) to 15.5±0.85 (n=6)) and 4.5-fold during 24 hours (from 9.66±0.92 (n=6) to 43.4±1.47 (n=6)) (Figure 1). When sodium ascorbate was added to the SMG culture, the number of buds was increased by approximately 60% at 2.27 mM and declined at higher concentrations, regardless of the incubation time (12 or 24 hours) (Figure 2).

Effect of EGCG on the growth of SMG. When control SMG cultures were incubated in serum-free BGJb medium, the number of buds in the control SMG cultures was increased 2.5-fold during the first 12 hours (from 5.50±0.50 (n=5) to 13.5±1.91 (n=5)) and 7.9-fold during 24 hours (from 5.50±0.50 (n=5) to 43.4±1.47 (n=5)) (Figure 3). When EGCG was added to the SMG culture, the number of buds was decreased in a dose-dependent manner and epithelial growth was significantly suppressed at the highest concentration (220 μM) (Figure 4).

Discussion

BGJb medium has been extensively used for the culture of SMG rudiments. Since this medium contains ascorbic acid (0.28 mM), we used ascorbic acid-free DMEM medium to investigate the effect of ascorbic acid on the growth of SMG rudiments. For most of the organ cultures of salivary gland tissue in DMEM, ascorbic acid (0.56 mM) and fetal bovine serum (FBS) have been included in the culture medium. As far as we know, however, there has been no report that investigates the effect of the addition of ascorbic acid to the SMG organ culture. The present study demonstrated that the addition of insufficient amounts of sodium ascorbate (0.25-0.76 mM) to DMEM did not significantly stimulate the growth of SMG. This indicates that the previous use of 0.56 mM ascorbate would fail to stimulate the SMG branching morphogenesis. The addition of sodium ascorbate at 2.27 mM induced maximal stimulation of epithelial

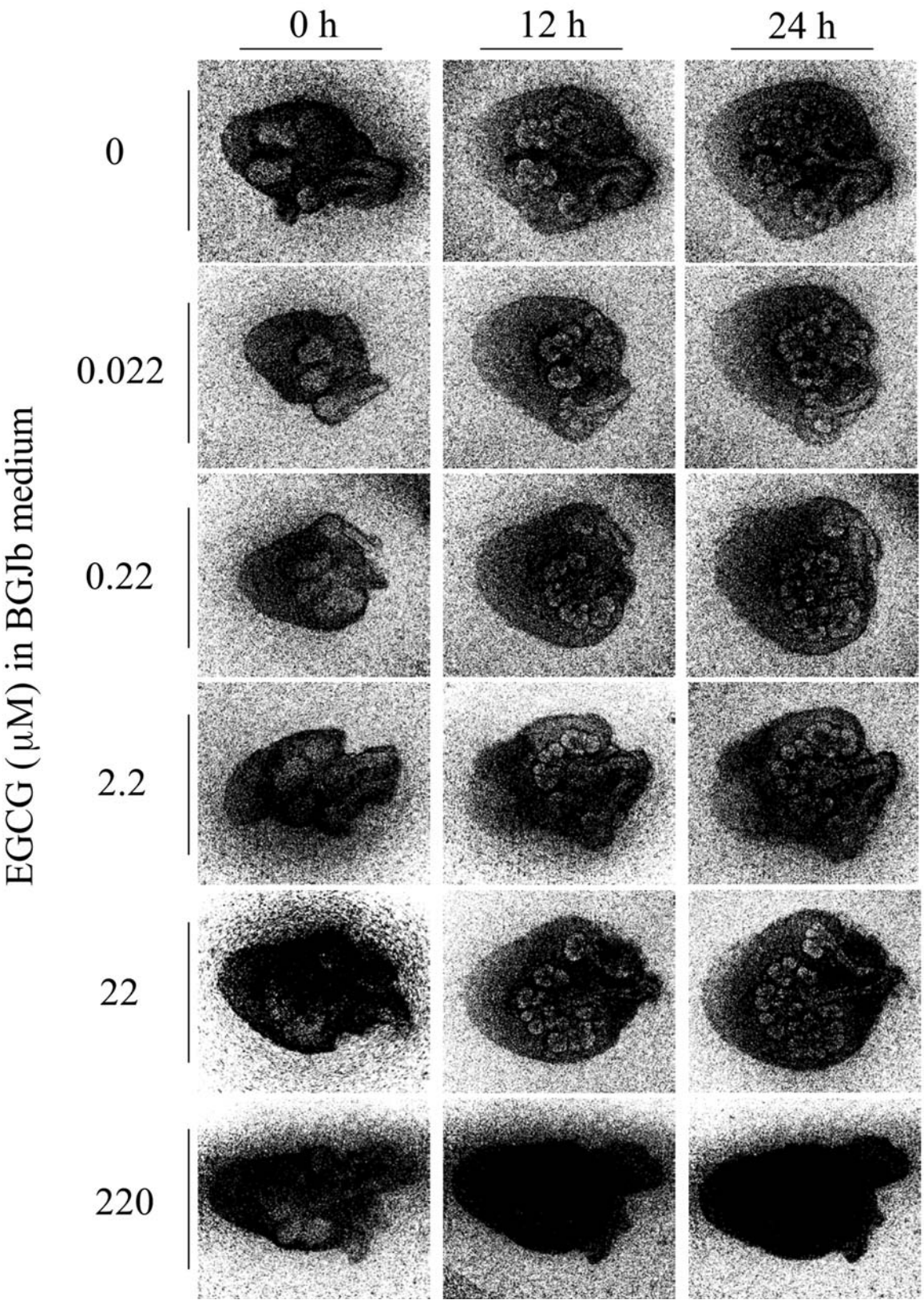

Figure 3. *Morphological changes induced by EGCG in organ culture of SMG. SMG from E13 mouse embryo was cultured for 0, 12 or 24 hours with the indicated concentrations of EGCG and photographed under a photomicroscope.*

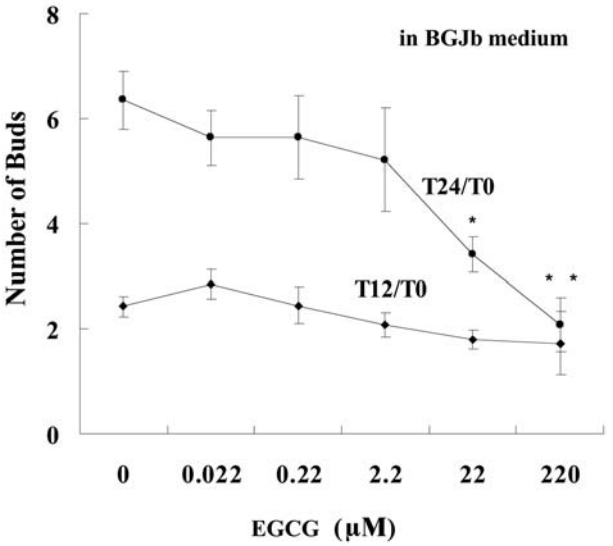

Figure 4. *Dose-effect of EGCG on the branching morphogenesis of SMG. SMG was cultured as described in Figure 3, and the buds in the SMG were counted after short-term (12 hours) and long-term (24 hours) incubation. The extent of growth of SMG was expressed as the ratio of the number of buds at 12 or 24 hours / the number of buds at 0 hour. Each value represents mean±S.E. from 5 determinations. *p<0.05, **p<0.01 vs. control.*

branching, possibly due to the promotion of collagen fiber production. This stimulatory effect of sodium ascorbate could be observed as early as 12 hours after treatment, suggesting that its stimulation effect may be expressed at a relatively early stage of SMG branching morphogenesis.

In contrast, EGCG, at wide ranges of concentration (0.22-220 μM), did not show any significant stimulation of SMG branching morphogenesis. EGCG rather reduced the SMG development dose-dependently. The inhibitory effect of EGCG was observed as early as 12 hours after EGCG treatment, suggesting the relatively early onset of its inhibitory action. It was found that addition of EGCG (220 μM) resulted in the disappearance of the contrast between epithelial and mesenchymal tissues, and blackening of the mesenchymal tissue. This suggests that the inhibitory effect of EGCG may reach both epithelial and mesenchymal tissues.

The present study also demonstrated, for the first time, that sodium ascorbate bi-modally affected the SMG branching morphogenesis. Sodium ascorbate, at lower concentrations, stimulated the SMG branching morphogenesis, whereas it was inhibitory at higher concentrations. This indicates that sodium ascorbate induces hormesis (a beneficial action at lower concentration (25)) during SMG branching morphogenesis. In contrast, EGCG did not show such a hormetic action, suggesting that not all antioxidants induce hormesis in the present model of SMG branching morphogenesis.

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