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Effects of Kimchi Extract on the Development of Multicellular Structures from Rat Mammary Organoids Cultured in Matrigel

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Abstract

The effects of methanol soluble fraction(MSF) of kimchi on the proliferating and differentiating activity of normal rat mammary epithelial cells or organoids in culture were studied. Reconstituted basement membrane, Matrigel, supported the growth and development several different multicellular structures from mammary organoids. The five type colonies of multicellular structures, stellate, ductal, webbed, squamous, and lobulo-ductal colonies, were observed in Matrigel culture. In methanol extract treated groups, webbed colonies were more and squamous colonies were less than control group. And the lobulo-ductal colonies which is known that it formed in welldifferentiated mammary epithelial cells were developed more in MSF treated group than control group. These results showed that methanol extract of kimchi affected on the proliferation and differentiation of normal rat mammary epithelial cells cultured in serum free medium condition.

Key words: kimchi extract, mammary epithelial cells, Matrigel, squamous metaplasia

INTRODUCTION

Kimchi is one of the most popular traditional fermented foods in Korea. About 187 different types of kimchi have been known(1). Kimchi is made of almost all kinds of vegetables, condiments, fishes, etc. It is nutritious and contains high levels of vitamins A and C, β-carotene, vitamin B complex, minerals of Ca, Fe, P and lots of dietary fibers. It also contains high levels of organic acids and lactic acid bacteria(2,3).

Recently, several properties of kimchi extracts were reported, such as antimutagenicity and anticancer effects. Antimutagenic activities of kimchi was studied with methanol extract of kimchi toward aflatoxin B_1(AFB_1), 4-nitroquinoline-1-oxide(4-NQO), and N-methyl-N'-nitro-N-nitrosoguanidine(MNNG) in the Ames test and SOS chromotest(3-6). The mutagenicities mediated by AFB_1, 4-NQO, and MNNG were significantly reduced when the methanol extracts of kimchi added at the levels of 1% and 5% in the Ames test. The inhibition rates of the mutagenicities were the highest at three-week fermented samples.

Recently, the antimutagenic and anticancer effects of four different kinds of *baechu* kimchi extracts were studied with methanol extract, hexane extract, and methanol soluble fraction(MSF) and kimchi juice *in vitro* and *in vivo*(7). The hexane extract showed stronger antimutagenic activity to aflatoxin B_1 on *Salmonella typhimurium* TA98 than any other kimchi extracts and juice. However, antimutagenicity test using aflatoxin B_1 on *Drosophila melanogaster* showed that small and large *mwh* spots were represented less in MSF treated group than in any other groups, suggesting that MSF significantly suppressed the mutagenicity of aflatoxin B_1.

Reconstituted basement membrane(RBM) supports the functional and morphological development of a variety of primary epithelial cells in culture(8,9). RBM has been used to support and promote mammary epithelial growth and differentiation, and even three-dimensional tissue organization and function(10,11). There was marked functional differentiation of rat mammary epithelial cells(RMEC) cultured in a RBM, Matrigel(12). Matrigel supports the growth and development of several different multicellular colonies from mammary organoids and from monodispersed epithelial cells in culture. Several types of colonies are observed including stellate colonies, duct-like structures, two- and three-dimensional web structures, squamous organoids, and lobulo-duct colonies(12). Squamous colonies are comprised of several layers of squamous epithelium surrounding keratin pearls

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as is typical of squamous metaplasia. All-*trans*, 13-*cis*, and 9-*cis* retinoic acid inhibited development of squamous metaplasia from rat mammary cells or organoids cultured in Matrigel in serum medium and partially inhibits squamous metaplasia in cultures in Matrigel under serum-free medium(13,14).

We studied the effects of methanol soluble extract of kimchi on the developments of multicellular structures, especially squamous metaplasia from rat mammary organoids cultured in Matrigel in serum free medium.

MATERIALS AND METHODS

Preparation of kimchi

The *baechu*(Chinese cabbage), garlic, ginger, red pepper powder, salt fermented anchovy and salt were purchased from the Onchun market in Pusan, Korea. The *baechu* kimchi were prepared as described previously. Briefly, *baechus* were cut into 8 pieces and soaked in 10% NaCl solution for 10hrs at room temperature and then rinsed with tap water. The compositions of ingredients for the kimchi preparation are shown in Table 1. The prepared kimchi(0 week) was put into pint jars, and packed tightly. The rid of the jar was closed tightly and then fermented at 5°C for six weeks.

Extraction and fractionation of kimchi sample

After fermentation at 5°C for three weeks, kimchi samples were chopped and freeze dried. For methanol extract, about 20 folds of methanol(50g dried kimchi/1 L) was added to the dried powdered samples and extracted three times. The methanol extracts were evaporated using vacuum evaporator(Büchi RE121, or 100, Switzerland), concentrated and then dissolved in 1% dimethylsolfoxide(DMSO, Aldrich Chemical Co., USA) (Fig. 1). For hexane extract, about 20 folds of hexane was added to the dried powdered samples and extracted three times. The hexane extracts were evaporated using vacuum evaporator, concentrated and then dissolved in 1% DMSO. For methanol soluble fraction, the residues of hexane extract were further extracted with stirring in about 20 folds of methanol, heated for 90min, filtered with Whatman filter paper, evaporated, concentrated and then dissolved in 1% DMSO. The procedure is summarized as in a scheme(Fig. 1).

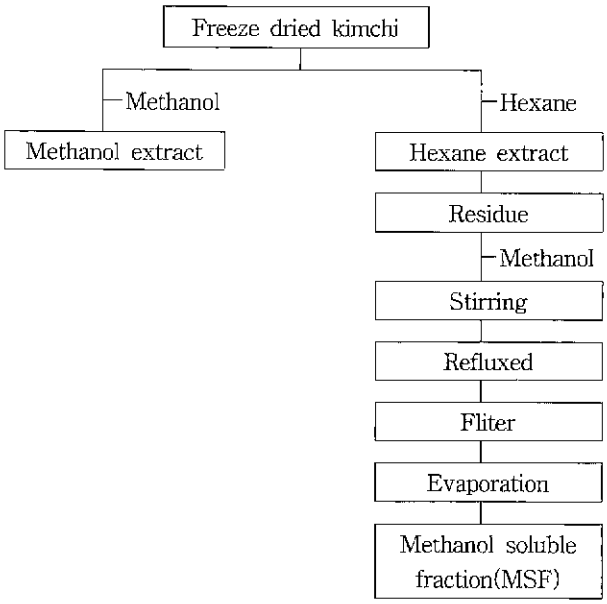

Fig. 1. A scheme for the preparation of kimchi extracts.

Table 1. Compositions of ingredients for the kimchi preparation

Ingredient	Kimchi(w/w)
Chinese cabbage	100%(3.000g)
Red pepper powder	2%(60g)
Crushed garlic	2%(60g)
Crushed ginger	0.5%(15g)
Final salt concentration	3.0%

Rat mammary epithelial organoids preparation

Mammary epithelial organoids were prepared as described previously(15,16). Briefly, virgin female F344 rats, 50~55 days old, were killed with exposure to overdosed CO_2 gas, and their inguinal mammary fat pads were removed, and scissors-minced, and then digested with collagenase solution(Type III, 2mg/ml, Worthington Biochemical, Freehold, NJ, USA) in serum free medium (SFM, serum free Dulbecco's Modified Eagle Medium, added with 50μg/ml gentamycin sulfate and with 0.33 mg/ml glutamine, Gibco, Grand Island, NY, USA). After digestion, the suspension was washed in serum medium (SM : SFM with 10% fetal bovine serum, FBS, HyClone, Logan, UT, USA) and centrifuged. The pellet which containing cells, cell clumps, and mammary organoids was collected. The pellet was washed, resuspended, and passed onto a 40μm pore nylon mesh filter (Tetko, Brearcliff Manor, NY, USA) which allowed only the dispersed cells and small cell clumps to pass. The trapped organoids on the filter surface were col-

lected and washed with medium. The numbers of these organoids were then calculated under microscope. These organoids were consisted with mammary epithelial cells and ducts.

Mammary epithelial organoid cultured in Matrigel

Aliquots of 400μl of Matrigel were mixed with 100μl of SFM containing ~100 organoids and immediately distributed into 24 multi-well Primaria tissue culture plates(Falcon, USA). The plates were then incubated at 37°C for 30min to allow gel formation. The sample groups treated one ml medium containing 0.5%, 1.0%, and 2.0% MSF and a control group which was not treated with MSF were cultured for four weeks in serum-free mammary epithelium growth medium(MEGM) based on MCDB 170(Mammary Epithelium Basal Medium, MEBM, Clonetics, USA). The MEGM consisted of MEBM supplemented with EGF(10ng/ml, Sigma), insulin (5.0μg/ml, Sigma), hydrocortisone(0.5μg/ml, Sigma), human transferrin(10μg/ml, Sigam), and gentamicin(50μg/ml, Sigma). The medium was changed every 3 days. Multicellular structures were observed every 2 or 3 days by phase contrast microscope, and made an observation for proliferation of the cells and took a picture when it was needed.

Examination of multicellular structures

After 4 weeks culture, the Matrigels obtained from the culture plate were washed with PBS, fixed in methanol/chloroform/acetic acid(6 : 3 : 1) at 4°C overnight, routinely processed, and embedded in paraffin. By the standard method for tissue specimen treatment, paraffin blocks containing the gel were made and cut by microtomb with thickness of 5~6μm. The sections were stained with hematoxylin and eosin and then observed with optical microscope.

Statistics

Statistical significance was determined by Student's t-test. $p<0.05$ was judged to be statistically significant.

RESULTS AND DISCUSSION

In this current experiments, we certified that 0.5%, 1%, and 2% MSF in culture medium have slightly influenced on the development of multicellular structures from rat mammary organoids cultured in Matrigel. The mammary epithelial organoids distributed into 24 multiwell Primaria tissue culture plates were cultured in 37°C incubator for 4 weeks. As the results of the culture, multicellular structures were formed(Fig. 2). The mammary organoids were developed into several morphologically different multicellular structures with time. The multicellular colonies were classified into five types, based on their general appearance at the light microscopic level : ductal, webbed, stellate, squamous and lobulo-ductal colonies(Fig. 2A-E).

The numbers of developed colonies of control, 0.5%, 1.0%, and 2.0% groups were 82.00±5.00, 77.33±9.50, 72.67±7.77, and 68.33±7.57, respectively(Fig. 3). With the augmentation of MSF concentration, the total numbers of developed multicellular structures were slightly decreased. However, there were no statistically significance among the groups.

Each multicellular colony types were classified as stated and compared the frequencies of them(Fig. 4).

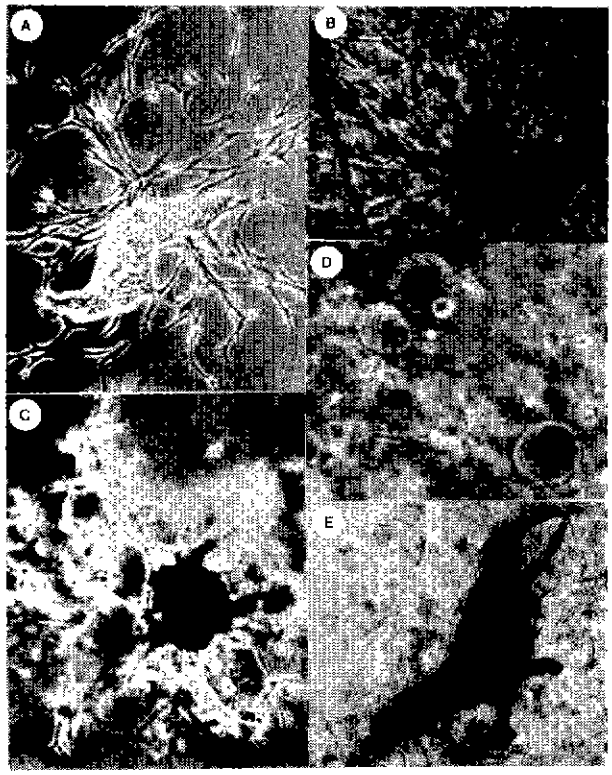

Fig. 2. Morphological appearance of mammary organoids cultured in Matrigel under serum-free MEGM conditions.
The resulting colonies were classified into one of five types : ductal colonies(A), stellate colonies(B), webbed colonies(C), squamous colonies(D), and lobulo-ductal colonies(E). ×100.

The frequencies of ductal and squamous colonies were reduced with the increase of MSF concentration. Especially, the frequency of squamous colony was significantly reduced. However, the frequencies of webbed and lobulo-ductal colonies were increased with the augmentation of MSF concentrations. There was no change in the frequency of stellate.

The most striking changes in response to MSF were the decrease in squamous colonies and the increase in the webbed and lobulo-ductal colonies. Webbed colonies which were supposed to be developed from myoepithelial cell were more than control groups in sample groups. Squamous colony formation from organoids in SFM decreased with increasing MSF concentrations. However, the squamous colony formation from organoids was not totally suppressed as in retinoic acid(15).

The stained sections of the gels with hematoxylin and eosin were identified that these structures were identical to those which we had been studied(data not shown). Lobulo-ductal colonies secreted casein, lipids and so on. Stellate and webbed colonies were composed of cell groups with foamy cytoplasms. Squamous colonies which had keratin pearl and multilayers of keratin around the center of the colonies were squamous metaplasia(Fig. 5). The squamous metaplasia from the results of dedifferentiation of mammary epithelial stem cell were markedly reduced in MSF treated sample

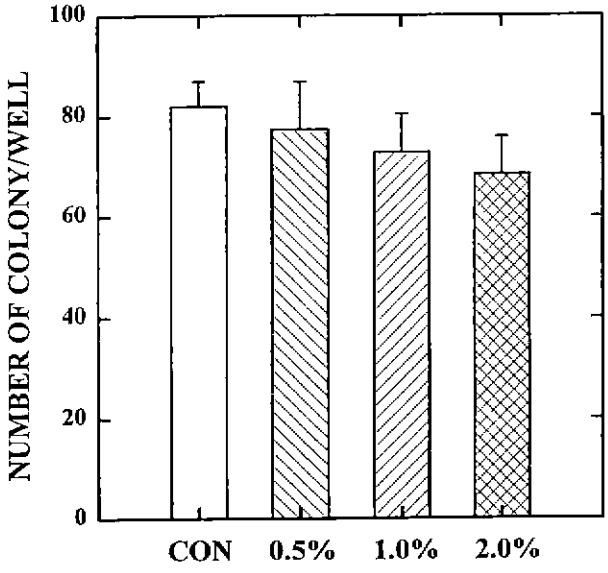

Fig. 3. The effects of MSF of kimchi on the total numbers of colonies developed from rat mammary organoids grown in serum-free MEGM.
Morphology was quantitated during Days 28 of cultures. Bars represent the mean±SD of colonies per well of triplicate wells.

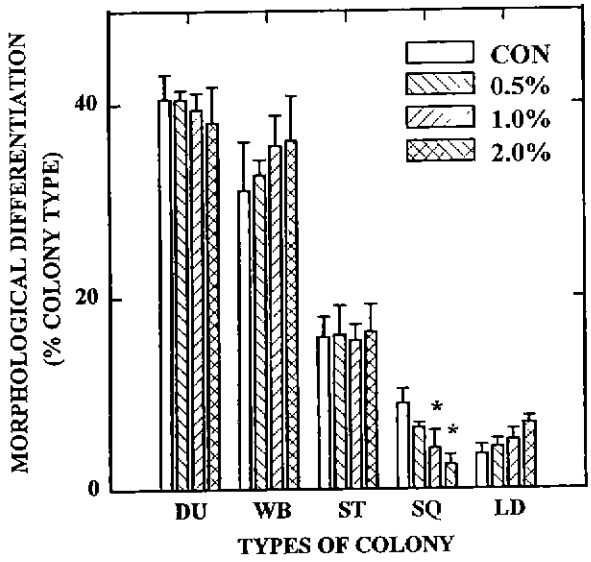

Fig. 4. The effects of MSF of kimchi on morphological differentiation of normal rat mammary organoids grown in serum-free MEGM.
Morphology was quantitated during Days 28 of cultures. Ductal(DU), webbed(WB), stellate(ST), squamous(SQ), or lobulo-ductal(LD) colonies. Bars represent the mean±SD of colonies per well of triplicate wells. Statistical significance' * $p<0.05$ vs. control group.

Fig. 5. Morphological appearance of the squamous metaplasia in organoids cultured in serum-free MEGM.
Histological section of squamous metaplasia was stained with H & E. Squamous structures were composed of a multilayered cells with a keratin pearl(KP) at center. ×400.

groups. The lobulo-ductal colonies, which is known to form in well-differentiated mammary epithelial cells, were developed more in MSF treated groups. These results represent that some ingredients in MSF have epithelial cell differentiation inducing activity and have an important role in the induction of normal differentiation of squamous metaplasia.

Squamous metaplasia results from aberrant differentiation of one cell phenotype into a differentiated phenotype with characteristics typical of a related tissue (16). Squamous metaplasia is observed *in vivo* in response to chronic injury or vitamin A-deficiency in several organs including the salivary and prostate glands and the respiratory and olfactory epithelia(16-21). Schaefer et al.(22,23) induced squamous differentiation in human breast cultures and in preneoplastic mouse mammary outgrowths by exposure to cyclic adenine nucleotide.

Retinoic acid and related compounds play important regulatory roles in growth and differentiation of a wide variety of cell types. Indeed, retinoids are required to maintain normal differentiation and proliferation of epithelial tissues in general. The regulation of squamous differentiation by retinoids has been extensively investigated using primary tracheal epithelial cells(24) and rat mammary epithelial cells(15). Therefore, some ingredients in MSF, such as vitamin A or its derivatives, may have affected on the depression of squamous metaplasia formation. However, more experiments are needed to be clarified.

Organoids have the greatest proliferative potential and formation of multi-cellular structures. Phase contrast micrographs demonstrate extensive intracellular lipid accumulation within the web structures and some of duct-like colonies(12). At the immunocytochemical and electron micrograph level, casein proteins are predominantly localized near the apical surface of the cells or in the lumen of duct-like or lobulo-duct colonies. The current model system may allow further systematic investigations of the effects of a variety of hormones, growth factors, and bioactive compounds on *in vitro* morphogenesis of mammary epithelial cells and on mechanisms of aberrant differentiation such as squamous metaplasia formation.

In summary, MSF did not show any toxic effects on the growth of normal, intact mammary organoids cultured in Matrigel in serum-free MEGM and partially inhibited squamous metaplasia in cultures. This system may serve as an useful tool not only for understanding cell growth and differentiation of epithelial cells but also for the isolation and characterization of differentiation inducing agents.

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