

**Anti-proliferative effects of  
b-irradiation on estrogen-treated  
vascular smooth muscle cells**

Thesis by  
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**Anti-proliferative effects of  
b-irradiation on estrogen-treated  
vascular smooth muscle cells**

Directed by Professor Yangsoo Jang

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A Dissertation for the Degree of Master in Medical  
Sciences by Dae Keun Shim has been approved by

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June 1, 2001

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## Abstract

# Anti-proliferative effects of $\beta$ -irradiation on estrogen-treated vascular smooth muscle cells

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(Directed by Professor Yangsoo Jang)

Restenosis following percutaneous transluminal coronary angioplasty is characterized by significant neointimal formation and geometric remodeling of the artery. Estrogen and radiation inhibit cellular proliferation and migration through many effector mechanisms *in vitro*, and inhibit neointimal proliferation after vessel injury *in vivo*. Extracellular signal and stress are introduced intracellularly via diverse intracellular signal pathways to finally produce cell proliferation, apoptosis, differentiation, cytoskeleton remodeling, and cell cycle alteration. The mitogen-activated protein kinases (MAPK) have been shown to play an important role in the transduction of extracellular signals into cellular responses. The anti-proliferative effects of estrogen and radiation were expected to influence this novel signal pathway.

<sup>166</sup>Holmium(Ho)-DTPA was used as a radiation source. The desired absorbed dose was calculated by dosimetry. Rat aorta smooth muscle cell (RASMC) proliferation

was determined using the trypan blue dye-exclusion method using a hemocytometer. The expression and activity of MAPK cascades were evaluated by western blot. The proto-oncogene analysis of *c-fos*, *c-jun*, and *elk-1* mRNA levels was carried out by RT-PCR.

Estrogen and <sup>166</sup>Ho-DTPA inhibited cell proliferation in a dose dependent manner. Estrogen inhibited RASMC proliferation through the selective inhibition of the extracellular signal-regulated kinases (ERK) of the MAPK signal, which was followed by the suppression of MAP kinase-kinase (MEK)1,2, and led to the down-regulation of *elk-1*. However, <sup>166</sup>Ho-DTPA did not show the synergistic effects with estrogen on ERK inactivation, which indicates that the anti-proliferative mechanisms of Ho-166 and estrogen are distinct in RASMCs. Although radioactivities of <sup>166</sup>Ho-DTPA have diverse influences on many subcellular mechanisms, the anti-proliferative effects of <sup>166</sup>Ho-DTPA may be predominantly associated with cell cycle interruption rather than MAPK inactivation.

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**Key Words:** Rat aorta smooth muscle cells. Estrogen.  $\beta$ -Irradiation.

<sup>166</sup>Ho-DTPA. Mitogen-activated protein kinases

# Anti-proliferative effects of $\beta$ -irradiation on estrogen-treated vascular smooth muscle cells

<Directed by Professor Yangsoo Jang>

*Brain Korea 21 Project for Medical Sciences*  
*The Graduate School of Yonsei University*

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## **I. Introduction**

Percutaneous transluminal coronary angioplasty (PTCA) is one of the most common therapies for obstructive coronary artery disease. However, its success is limited by a 30% to 50% restenosis rate<sup>1,2</sup>. This is largely mediated by cell proliferation and extracellular matrix synthesis by modified smooth muscle cells that migrate in response to mechanical stretching and disruption at the site of the balloon angioplasty, as well as the overall vascular remodeling. The development of the neointimal component of the restenosis lesion is the result of a healing process initiated by vascular injury<sup>3</sup>. Despite numerous trials with pharmacological agents, including antiplatelet drugs, anticoagulants, corticosteroids, calcium-channel blockers, fish oils, and others, the frequency of restenosis has not diminished<sup>4,5</sup>.

Vascular smooth muscle cells (VSMCs) do not normally display actively dividing

cell populations. However, mechanical injury or other stimuli can induce a smooth muscle cell response characterized by migration, proliferation and matrix synthesis. Platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) released from platelets, and activated vascular cells are all considered important in the triggering of smooth muscle cell proliferation and migration<sup>6</sup>.

Ionizing radiation affects self-renewing tissues by arresting cell division, and therefore limits proliferation by reducing the number of clonal progenitors<sup>7</sup>. In this situation, ionizing radiation may effectively inhibit smooth muscle cell proliferation, and has been shown to inhibit thymidine uptake and collagen synthesis by cultured fibroblasts<sup>8,9</sup>. Several investigators have demonstrated that  $\gamma$ -irradiation, applied using an endovascular after-loading technique, can successfully inhibit neointimal proliferation in a porcine coronary injury model<sup>10,11</sup>. These investigators used a <sup>192</sup>Ir source to deliver an endovascular dose and demonstrated a dose-response relationship. Intra-arterial  $\beta$ -irradiation and a radioactive stent have also been shown to be effective at inhibiting neointimal proliferation<sup>12,13</sup>. However, if the radiation dose is too high, aneurismal dilatation may occur in a weak arterial wall, and if the dose is too low, VSMC proliferation will not be effectively inhibited. A further alternative involves the use of radioactive liquid-filled balloons<sup>14,15</sup>. Radioactive liquid-filled balloons may be superior to radioactive stents or radioactive catheters because they allow radiation to be applied homogeneously to the vessel walls surrounding angioplasty balloon. Though the biologic effect of the radiation on neointimal proliferation depends on the absorbed dose and not on the specific type of isotope,  $\beta$ -emitters have some obvious advantages over  $\gamma$ -emitters. The activity required to deliver a comparable dose is much lower for  $\beta$ -emitters and a special radiation shield

is unnecessary as  $\beta$ -emitters have limited penetration in tissue, and do not present undue radiation hazard concerns to patients or staffs. However, because of their limited range in tissue, centering is required in the endovascular use of  $\beta$ -emitters. But, if a  $\beta$ -emitting liquid-filled balloon could be used, labored centering and possible eccentric effects of the radiation source would not be a problem, because accurate source positioning and a uniform dose delivery to the vessel wall are virtually assured<sup>16</sup>.

Estrogen also exerts effects on vascular function and the structure of the vessel wall, and is involved many cellular and molecular mechanisms<sup>17</sup>. The protective effects of estrogens against cardiovascular disease in postmenopausal women are well documented. Estrogen has also been reported to have an anti-atherogenic action and to improve lipid metabolism by increasing high-density lipoprotein cholesterol levels and decreasing low-density lipoprotein cholesterol levels in serum. In addition, estrogen appears to have a vasodilatory action<sup>18</sup>, through a calcium-antagonistic effect and a regulatory effect upon prostacyclin and nitric oxide production<sup>19,20</sup>. Foegh et al. reported that estrogen can prevent myointimal proliferation and Moskowitz showed that estrogen also inhibits diet-induced atherosclerosis in rats and rabbits<sup>21,22</sup>. Currently, estrogen has been shown to inhibit the migration and proliferation of VSMCs *in vitro* and *in vivo*<sup>23,24</sup>. Roberto Vagas reported that estrogen inhibits thymidine uptake and smooth muscle proliferation in animal model, possibly via an estrogen receptor mechanism<sup>23</sup>. Other investigators also demonstrated that estrogen significantly decreases neointimal thickness by 50% to 70% in rabbits after balloon injury, by inhibiting myointimal response, demonstrating that this effect of estrogen is likely to be mediated directly by the inhibition of vascular smooth muscle cell

proliferation<sup>25</sup>. Estrogen inhibits cuff-induced intimal thickening, while inhibiting the migration and proliferation of rat VSMCs in culture<sup>26</sup>. In experiments by Do Dai-Do et al, estrogen specifically interfered with the effects of growth factors in VSMCs, regardless of gender. In addition to proliferation, the migration of VSMCs induced by growth factor was also effectively inhibited by estrogen<sup>27</sup>.

DNA damage and the biochemical alterations inflicted by radiation and other adverse external stimuli are recognized by a complex cellular sensing system, which leads to a number of possible effector pathways, such as DNA repair, cell cycle arrest, programmed cell death or genetic instability. The mitogen-activated protein kinases (MAPKs) pathway is one of the effector pathways activated by growth factors, cytokines and external stress. MAPKs, in turn, activate nuclear substrate and other kinases in a cascade of phosphorylation to finally produce cellular growth, differentiation, cytoskeleton remodeling, cell cycle interruptions and apoptosis<sup>28,29</sup>. The MAPKs play an important role in the transduction of extracellular signals into cellular responses. Several MAPK pathways have been described in mammalian cells, including the extracellular signal-regulated kinases (p42/44<sup>ERK</sup>)<sup>30</sup>, the c-jun N-terminal kinases (p46/54<sup>JNK</sup>)<sup>31</sup> and p38<sup>MAPK</sup><sup>32</sup>. These MAPK subfamilies respond in a different manner to a given stimulation. P42/44<sup>ERK</sup> is activated by growth factors and is involved in both cell proliferation and differentiation, whereas p46/54<sup>JNK</sup> and p38<sup>MAPK</sup> are related to inflammatory responses, cell cycle arrest, DNA repair and programmed cell death. MAPKs are activated by phosphorylation on Thr and Tyr by dual-specificity MAP kinase-kinases (MEKs). It has been reported that MEKs are, in general, very specific for downstream MAPKs<sup>33</sup>. MEK1 and MEK2 selectively phosphorylate and activate p42/44<sup>ERK</sup> subgroups, whereas MEK3 and MEK6

selectively activate p38<sup>MAPK</sup>. MEK4 also activates both p38<sup>MAPK</sup> and p46/54<sup>JNK</sup>, but not the p42/44<sup>ERK</sup> subgroup. Radiation and estrogen as applied external stresses upon cells could be possible activators of these well-known pathways and be activated predominantly in a certain pathways of MAPK subfamilies.

In this study, the anti-proliferative effect of liquid Ho-166 was evaluated as a  $\beta$ -emitter source and a promising filling agent for a radioactive balloon, and the anti-proliferative effect of estrogen on VSMCs was also evaluated in culture. To elucidate the molecular mechanisms of estrogen and/or radiation on the anti-proliferation of VSMCs, the influence on different MAPKs in VSMCs was investigated. Furthermore, the potential upstream activators of MAPKs, namely ras, protein kinase C (PKC) and MEKs and the expression levels of the protooncogenes, *c-fos*, *c-jun* and *elk-1*, involved in phosphorylation of MAPKs, were examined.

## **II. Materials and Methods**

### **1. Isolation and culture of VSMCs**

Rat aorta smooth muscle cells (RASMCs) were isolated by a modification of the method developed by Chamley-Campbell<sup>34</sup>. The thoracic aortas of 6-8 week-old Sprague-Dawley rats were removed. The aortas were free from connective tissue, transferred individually into petri dishes containing 5 mL of an enzyme dissociation mixture containing 1 mg/mL of collagenase type I (Sigma, Deisenhofen, Germany) and 0.5 mg/mL elastase (Sigma, Deisenhofen, Germany). Dishes were then incubated for 30 min at 37 °C. The adventitia was peeled away with a forceps under a binocular microscope, and the aorta was transferred to a plastic tube containing 5 mL of the enzyme dissociation mixture and incubated for 2 hours at 37 °C. The suspension was centrifuged (1500 rpm for 10 min) and the pellet resuspended with 10% FBS. Cells were then cultured over several passages (up to 10). RASMCs were maintained in complete medium containing 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin in 75-cm<sup>2</sup> flasks at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> (Forma Scientific, Inc., USA). For estrogen treatment, the cells were cultured in DMEM (Dulbecco's modified eagle medium) supplemented with 0.5% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 90% air and 10% CO<sub>2</sub> (Forma Scientific, Inc., USA).

### **2. Cell survival and proliferation assay**

The proliferative response of RASMCs was determined using a tetrazolium-based



colorimetric assay. Before all the experiments, confluent RASMCs were rendered quiescent by culturing them for 48 hours in 0.5% v/v FBS instead of 5% FBS. The assay is dependent upon the reduction of the tetrazolium salt WST-1 to the dark red formazan product by the various mitochondrial dehydrogenases of viable cells. Briefly, Premix WST-1 (10 mL/well) was added to cultured RASMCs in a 96-well culture dish ( $3 \times 10^4$ /well) and treated with a control medium or media containing the different concentrations of estrogen and liquid Ho-166. The cells were then further incubated at 37 °C for 4 hours in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The absorbances of the whole samples were measured at 450 nm using a microplate reader versus a background control. Cell viability was determined using the trypan blue dye exclusion method with a hemacytometer after 48 hours in the case of liquid Ho-166 treatment. For estrogen treatment, RASMCs were seeded in 24-well culture plates ( $2.5 \times 10^4$  cells/well; well diameter 12 mm), and further incubated in the DMEM medium containing 0.5% FBS for 48 hours. Cells were treated with estrogen (10-50 nM) and incubated for another 48 hours. Cells were then harvested from the plates using a 0.1% w/v trypsin solution, and cell viability was determined by the trypan blue dye exclusion test. The number of viable cells was estimated by microscopic cell counting using a hemacytometer.

### **3. Western immunoblot analysis**

At the end of the various treatments, cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, and 1 μg/mL leupeptin) for 15 min on ice. Soluble extracts were prepared by centrifugation at 16,000 g for 15

min at 4°C. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gel. After electrophoresis, proteins were electrotransferred to methanol-treated polyvinylidene difluoride membranes. The blotted membranes were washed twice with water and blocked by incubating with 3% nonfat dry milk in PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.5 g NaH<sub>2</sub>PO<sub>4</sub>, 0.2 g K<sub>2</sub>HPO<sub>4</sub> per liter) for 20 min at room temperature. For western blot analysis of the expressions and phosphorylation of ERKs (42 and 44 kDa), JNKs (46 and 56 kDa) and p38 MAPK, the membranes were probed with anti-ERK (anti-phospho-p42/44<sup>MAPK</sup>) antibody, anti-JNK (anti-phospho-p46/54<sup>JNK</sup>) and anti-p38 MAPK (anti-phospho-p38<sup>MAPK</sup>) antibody followed by peroxidase Goat anti-rabbit IgG and detected by ECL. For the western blot analysis of ras, PKC, and MEK1,2, antibodies against ras (21 kDa), PKC (82 kDa), and MEK1 (43 kDa) were used.

#### **4. RT-PCR analysis**

The expression levels of the proto-oncogenes, *c-fos*, *c-jun* and *elk-1* mRNA were analyzed using the reverse transcription polymerase chain reaction (RT-PCR) technique. For the RNA preparation, confluent RASMCs were cultured for 48 hours in DMEM containing 0.5% serum and treated with estrogen (10-50 nM) for 72 hours at 37 °C. Total RNA was prepared using the Ultraspect<sup>TM</sup>-II RNA system (Biotecx Laboratories, Inc., USA) and single-stranded cDNA was then synthesized from isolated total RNA by AMV reverse transcriptase. A 20 µl reverse transcription reaction mixture containing 1 µg of total RNA, 1X reverse transcription buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs), 0.5 unit of RNase inhibitor, 0.5 µg of oligo (dT)<sub>15</sub>, and 15

units of AMV reverse transcriptase was incubated at 42 °C for 15 min, heated to 99 °C for 5 min, and then incubated at 0-5 °C for 5 min. PCRs were performed for 35 cycles with each of the 3' and 5' primers based on the sequences of *c-fos* gene primers; 5' - accatgatgttctcgggttcaa-3' and 5' -ctctgtaatgcaccagctcagtc-3' ; *c-jun* gene primers; 5' - gaagtgaccgactgttctatgact-3' and 5' -cgcaaccagtcgaagtctcaagtt-3' ; *elk-1* gene primers; 5' -tacatcgtagcgaatcaatcaatccgtg-3' and 5' -tggtgggtagtagtcatggctt-3' . The actin primers (5' -catcacatactcacaacgctcaac-3' and 5' -catagcacgatggctgattgtcgt-3' ) were used as an internal standard. The signal intensities of amplification products were normalized to their respective actin signal intensities.

## **5. Quantitation of proteins**

Concentration of protein was determined with the Bio-Rad DC protein assay reagent (Bio-Rad Laboratories), versus an albumin standard.

## **6. Estrogen treatment**

The cells were cultured without and with estrogen at concentrations of 10, 20, 30, 40 and 50 nM. Phenol red and weak estrogen receptor agonist were absent from the culture medium. When the cells were 70 – 80% confluent, the culture media were replaced with Phenol red and serum-free DMEM medium containing 0.5% FBS. Estrogen was then added at the stated concentrations to each culture medium. After culturing for 48 hours, the effects of the estrogen upon cell proliferation were analyzed.

## **7. Radiation procedure**

Radioactive liquid Ho-166 (high energy  $\beta$ -emitter, pH adjusted to 7.0) was added to the culture media at 0, 1.25, 2.5, 5, 10, 15 and 20 Gy by changing Ho-166 radioactivities. The admixture containing the Ho-166 was washed out after a scheduled time for each absorbed dose, and replaced with fresh culture media and stimulated with 5 ng PDGF or 10% FCS. The effects of radiation were analyzed 12, 24 and 48 hours after liquid Ho-166 addition.

### 8. Dosimetry of liquid Ho-166

As a  $\beta$ -emitter available in liquid form, Ho-166 has unique features compared with the other high-energy  $\beta$ -emitters (Table1).  $^{32}\text{P}$ ,  $^{89}\text{Sr}$ ,  $^{90}\text{Y}$ ,  $^{165}\text{Dy}$ ,  $^{166}\text{Ho}$ ,  $^{186}\text{Re}$  and  $^{198}\text{Au}$  are inherent bone seekers and thus, when used as a filling agent for a radioactive balloon, balloon rupture would have a devastating outcome due to radiation damage to whole body and bone marrow. So, Ho-166 was labelled with DTPA (diethylenetriaminepentaacetic acid) to promote its urinary excretion in the event of balloon rupture.  $^{166}\text{Ho}$ -DTPA is excreted via the urinary system within 30 min and bone uptake is only 0.05% of injected dose after 90 min<sup>35</sup>.

The total radiation dose and the dose at intermediate times were calculated as follows :

$$D_{b+g} = C \cdot T \cdot (73.8 \cdot E_b + 0.0346 \cdot r \cdot g)$$

$$D_t = D_{to} \cdot (1 - e^{-0.693t/T})$$

C: Concentration ( $\mu\text{Ci/g}$ )

D: Absorbed dose (cGy)

$D_{to}$ : Total absorbed dose for complete decay

$D_t$ : Total absorbed dose for an intermediate time

$E_{\beta}$ : Mean  $\beta$  energy (MeV)

g: Geometric factor

$g$ : Gamma factor (specific gamma emission)

T: Half-life (days)

The amount of Gamma radiation in Ho-166 is only 4% of total radiation.

So,  $D_{\beta+\gamma}$  can be estimated to be  $D_{\beta}$  and  $D_t = D_b (1 - e^{-0.693t/T})$

The dose distribution of  $\beta$ -emission from liquid Ho-166 was calculated by Monte Carlo simulation using the EGS4 code system<sup>36,37</sup>. In the dosimetry calculations, liquid Ho-166 was assumed to be distributed evenly in the contained volume. Using window-based software and by taking the different times into account, the amount of radiation absorbed can be estimated as a function of time (Table 2). The radioactivity of manufactured liquid Ho-166 according to the dosimetry was re-estimated by calibrator (Capintec, U.S.A.) before experiment. The difference between the two radioactivities was 2.25 % as a mean (Table 3).

## 9. The combined effect of radiation and estrogen

RASMCs were pretreated with 40 nM estrogen for 48 hours and then were exposed to 0.3 Gy of Ho-166. The combined effects of Ho-166 and estrogen were analyzed 24 hours after incubation.

## 10. Data analysis

Results are presented as mean  $\pm$  SEM. Values within 95% confidence intervals ( $p < 0.05$ ) were considered significant.

### **III. Results**

#### **1. Effects of radiation on cell proliferation**

Cell proliferation was determined by measuring the amount of dark red formazan formed by mitochondrial dehydrogenases in RASMCs exposed to different radiation doses. The proliferation of RASMCs was inhibited by  $\beta$ -irradiation of liquid Ho-166 in a dose related fashion for radiation doses of 1, 2.5, 5, 10, 15 and 20 Gy with significant cell proliferation inhibition between 1 and 2.5 Gy, and 10 and 15 Gy (Figure 1). The IC<sub>50</sub> value was estimated at 2.5 Gy. These findings indicate that radiation inhibits cellular proliferation in a dose-dependent manner. Other investigators have also shown that, regardless of radiation source and method of application, radiation inhibits cell proliferation in a dose dependent manner. In this experiment, Ho-166 was used as a radiation source and cell proliferation was evaluated in an admixture of <sup>166</sup>Ho-DTPA and culture media. This effect of radiation on cell proliferation indicates that liquid <sup>166</sup>Ho-DTPA may also be an effective radiation source for cell proliferation inhibition, and that mixtures of <sup>166</sup>Ho-DTPA with cell culture media may be conveniently used as an irradiation method in cell culture systems without any direct cellular toxicity in itself.

#### **2. Effects of estrogen on cell proliferation**

Cell proliferation was determined by measuring the amount of dark red formazan formed by mitochondrial dehydrogenases in RASMCs at different estrogen concentrations (10 to 50 nM). The results show that estrogen inhibits proliferation in

a dose-dependent manner up to a dose of 50 nM of estrogen in 5% serum-stimulated RASMCs. Cell viability, as determined by the trypan blue dye exclusion method, was also affected in a dose-dependent manner (Figure 2). The  $IC_{50}$  value of estrogen was 40 nM. These results are also consistent with those reported for rabbit aortic SMCs<sup>38</sup>, rat vascular SMCs<sup>26</sup>, human female aortic SMCs<sup>39</sup>, and pig coronary artery SMCs<sup>23</sup>. These findings indicate that estrogen also inhibits the proliferation of RASMCs in a concentration-dependent manner within the physiological range ( $10^{-10}$  to  $10^{-8}$  M).

### **3. Effects of radiation and estrogen on MAP kinases activity**

To investigate the anti-proliferative mechanisms of estrogen and Ho-166 in RASMCs, the MAPK signaling responses for serum-stimulated proliferation were investigated. To characterize whether the anti-proliferative mechanisms of estrogen involved the MAPK signaling cascades, the activation of MAPK was determined by western blot with phosphospecific antibodies that recognize only the phosphorylated and active form of MAPK, at various estrogen concentrations in the 0.5% serum-treated RASMCs. Earlier studies have shown that estrogen differentially inhibited the MAPK pathway in various cells. In RASMCs, estrogen specifically inhibited ERK activity in a dose-dependent manner, even though the activities of JNK and p38<sup>MAPK</sup> were only slightly affected by the high concentrations (>100 nM) of estrogen (Figure 3). As p42/44<sup>ERK</sup> is involved in the promotion of both cell proliferation and differentiation, it is possible that estrogen inhibits cell proliferation via the ERK pathway. The inactivation of ERK was selectively followed by the suppression of the activity of MEK1,2 as an upstream regulator (Figure 4) and led to the suppression of the early gene *elk-1* transcription in a dose-dependent manner (Figure 5). These

indicate that MEK1,2 selectively phosphorylates and activates p42/44<sup>ERK</sup>, and that the MEK1,2/p42/44<sup>ERK</sup> cascade is coupled to the transcription of the *elk-1* gene in RASMCs. Based on these results, the activity changes of ERK in serum-stimulated RASMCs were investigated after estrogen (40 nM) and/or Ho-166 (0.3 Gy) treatment. As shown in Figure 6, the activity of ERK in serum-stimulated RASMCs increased compared to the serum-starved control, suggesting RASMCs were stimulated for cell proliferation. ERK activity was not increased by serum stimulation in RASMCs treated with estrogen (40 nM) and Ho-166 (0.3 Gy), which indicates that estrogen and Ho-166 influence MAPK pathway in a certain way. However, low dose of Ho-166 did not further decreased ERK activity in RASMCs pretreated with estrogen, which indicates that the combined effect of estrogen and Ho-166 did not cause synergistic inhibition of ERK activity. These results show that the main anti-proliferative mechanisms induced by estrogen and Ho-166 probably differ.



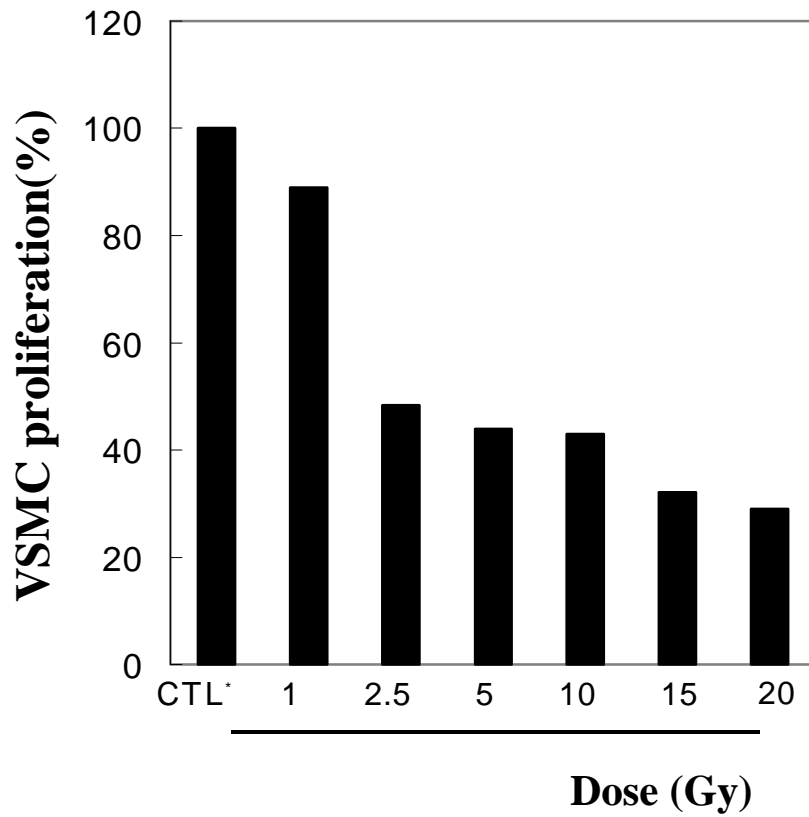


Figure 1. Dose-response effects of  $\beta$ -irradiation on cell proliferation of VSMCs. The proliferation of RASMCs was inhibited by  $\beta$ -irradiation of liquid Ho-166 in a dose related fashion with significant inhibition of cell proliferation between 1 and 2.5 Gy, and 10 and 15 Gy. The  $IC_{50}$  value was 2.5 Gy. CTL\*: control

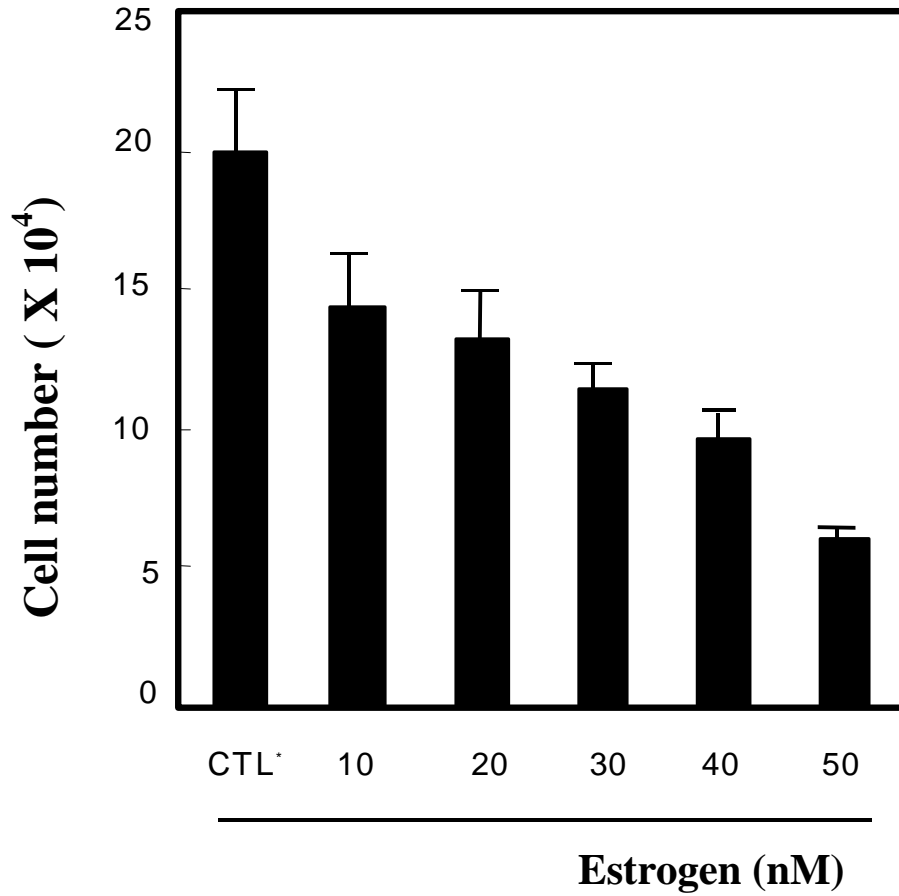


Figure 2. Estrogen inhibition of VSMCs. The results showed that estrogen inhibited cell proliferation in a dose-dependent manner up to 50 nM of estrogen in 5% serum-stimulated RASMCs. Cell viability, using the trypan blue dye exclusion method, was affected in a dose-dependent manner. The IC<sub>50</sub> value was 40 nM. CTL\*: control

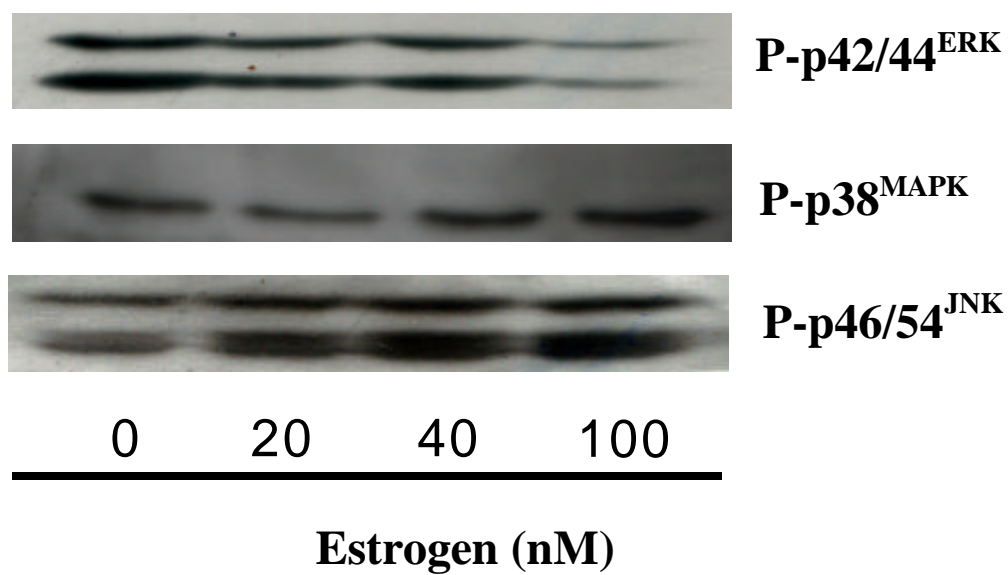


Figure 3. Estrogen effect on MAPK. In RASMCs, estrogen specifically inhibited ERK activity in a dose-dependent manner, even though activities of JNK and p38<sup>MAPK</sup> were only slightly affected by high concentration (>100 nM) of estrogen.

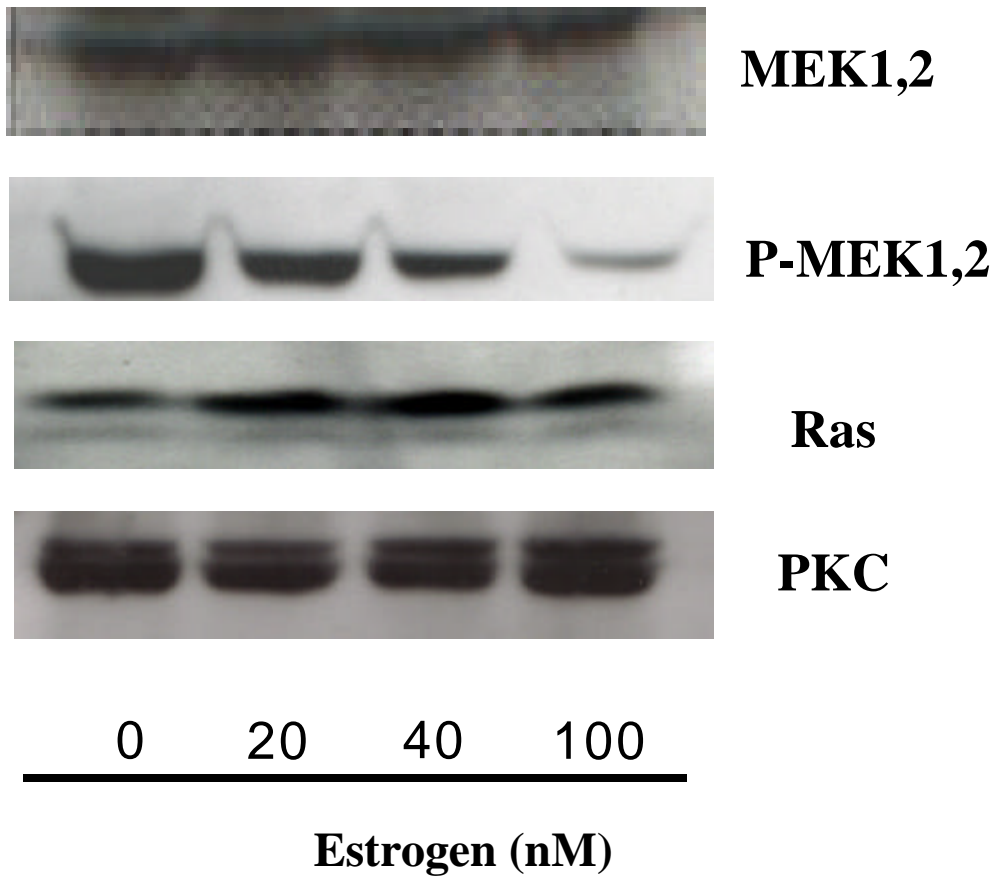


Figure 4. MEK1,2 suppression as an upstream regulator of ERK by estrogen. ERK inactivation was selectively followed by suppression of MEK1,2 activity in a dose-dependent manner. These indicate that MEK1,2 selectively phosphorylate and activate p42/44<sup>ERK</sup>.

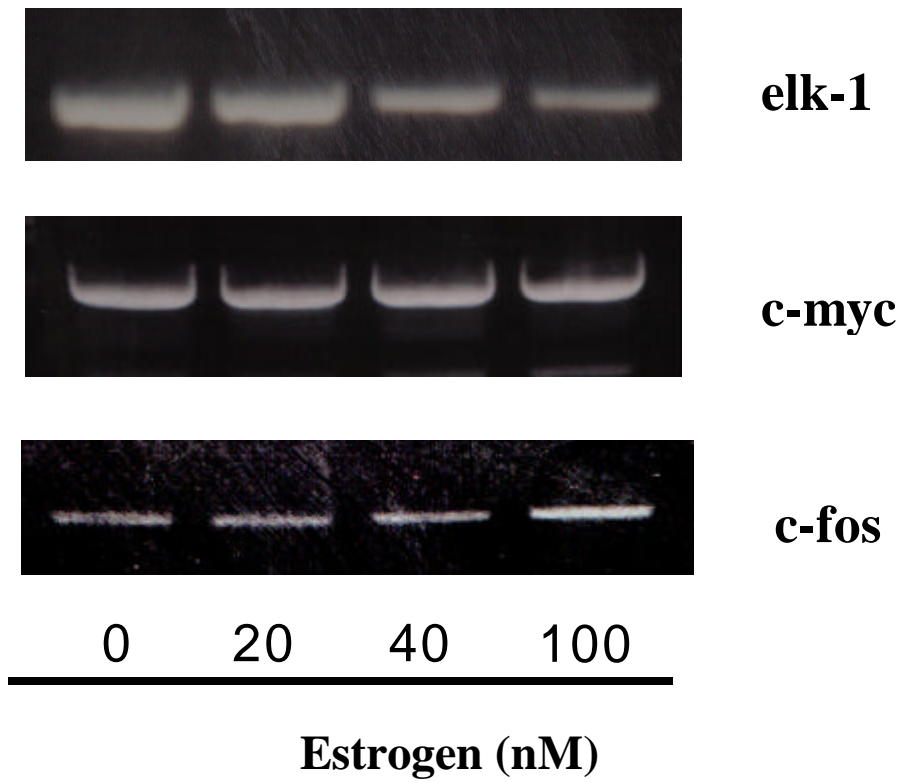


Figure 5. The selective inhibition of the early gene *elk-1* transcription. ERK inactivation led to suppression of the early gene *elk-1* transcription in a dose-dependent manner. The MEK1,2/p42/44<sup>ERK</sup> cascade is coupled to the transcription of *elk-1* gene in RASMCs.

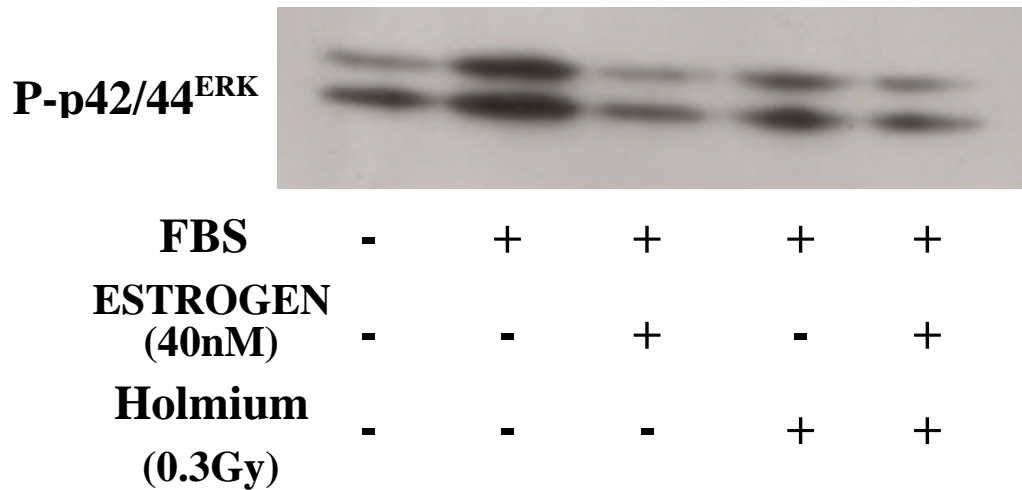


Figure 6. The combined effect of estrogen and Ho-166. ERK activity in serum-stimulated RASMCs was increased compared to the serum-starved control, suggesting increased stimulation for cell proliferation. However, ERK activity was not increased by serum-stimulation in RASMCs pretreated with estrogen (40 nM) and Ho-166 (0.3 Gy), which indicates that estrogen and Ho-166 influence on MAPK pathway in a certain way. The combined effect of estrogen and Ho-166 did not show further inhibition of ERK activities. These results indicate that the main anti-proliferative mechanisms induced by estrogen and Ho-166 probably differ.

Table 1. A Selection of  $\beta$ -emitters available in liquid form

Radionuclide	Radiation	Emax in MeV (% abundance)	Physical half-life	Max. penetration (mm)
P-32	Beta	1.7	14 days	7.9
Y-90	Beta	2.3	2.7 days	11.1
Ho-166	Beta	1.84	27 hours	8.4
	Gamma	0.08(4%)		
Re-188	Beta	2.2	17 hours	11.0
	Gamma	0.155(15%)		

Table 2. Absorbed radiation dose estimated as a function of time at various initial radioactivities (mCi/mL)

Sec	1.25 Gy	2.5 Gy	5 Gy	10 Gy	15 Gy	20 Gy
300	75.88451	151.769	303.538	607.0761	910.6141	1214.152
600	37.94225	75.88451	151.769	303.538	455.3071	607.0761
900	25.29484	50.58967	101.1793	202.3587	303.538	404.7174
1200	18.97113	37.94225	75.88451	151.769	227.6535	303.538
1500	15.1769	30.3538	60.70761	121.4152	182.1228	242.8304
1800	12.64742	25.29484	50.58967	101.1793	151.769	202.3587
2100	10.84064	21.68129	43.36258	86.72515	130.0877	173.4503
2400	9.485564	18.97113	37.94225	75.88451	113.8268	151.769
2700	8.431612	16.86322	33.72645	67.4529	101.1793	134.9058
3000	7.588451	15.1769	30.3538	60.70761	91.06141	121.4152
3300	6.898592	13.79718	27.59437	55.18873	82.7831	110.3775
3600	6.323709	12.64742	25.29484	50.58967	75.88451	101.1793
3900	5.83727	11.67454	23.34908	46.69816	70.04724	93.39632
4200	5.420322	10.84064	21.68129	43.36258	65.04387	86.72515
4500	5.058967	10.11793	20.23587	40.47174	60.70761	80.94348
4800	4.742782	9.485564	18.97113	37.94225	56.91338	75.88451
5100	4.463795	8.927589	17.85518	35.71036	53.56554	71.42072
5400	4.215806	8.431612	16.86322	33.72645	50.58967	67.4529
5700	3.993922	7.987843	15.97569	31.95137	47.92706	63.90275
6000	3.794225	7.588451	15.1769	30.3538	45.53071	60.70761

Doses were calculated for 0.1cc <sup>166</sup>Ho-DTPA in a total volume of 5cc



Table 3. Radioactivities of the calculated dose, and the calibrator reading and the difference between these two values

Absorbed Radiation Dose	Calculated Dose (mCi/mL)	Calibrator Read.(mCi/mL)	Difference	Differ./Calc. (%)
2.5 Gy	19.34094	18.99859	0.342348	1.8
	12.64742	13.01734	0.36992	2.9
	25.29484	24.44003	0.854804	3.4
5 Gy	38.68188	38.22906	0.452819	1.2
	25.29484	25.88569	0.59085	2.3
	37.94225	38.94833	1.00608	2.7
10 Gy	77.36375	75.93082	1.432935	1.9
	50.58967	51.58515	0.99548	2.0
	75.88451	72.95532	2.929189	3.9
15 Gy	116.0456	114.4235	1.622106	1.4
	75.88451	73.93251	1.951996	2.6
	113.8268	116.7285	2.90175	2.5
20 Gy	154.7275	150.7939	3.933606	2.5
	101.1793	102.3929	1.21355	1.2
	151.769	149.5584	2.210611	1.5 total 2.25%

## IV. Discussion

It is well established that restenosis after arterial injury is characterized by significant neointimal formation and geometric remodeling of the artery<sup>40</sup>. The pathogenesis of restenosis is multifactorial, but primarily involves the formation of intimal hyperplasia from proliferating smooth muscle cells<sup>41</sup>. To prevent neointimal hyperplasia, various therapeutic strategies have been developed based on pharmacological approaches, gene transfer, and more recently ionizing radiation using  $\beta$ - and  $\gamma$ -emitting sources. This study was undertaken to investigate whether estrogen and/or Ho-166 affect serum-stimulated proliferation and the subcellular mechanisms of RASMCs.

<sup>166</sup>Ho-DTPA in an aqueous mixture with VSMCs significantly inhibited cell proliferation in a dose dependent manner, giving an IC<sub>50</sub> at 2.5 Gy (Figure 1). These results are accord with those of other investigators in animal models. Specifically, these found that  $\gamma$ -emitters prevented restenosis over the short- and long-term and  $\beta$ -emitters also proved their effectiveness at reducing intimal hyperplasia in rabbit and pig models, respectively.

The property of anti-proliferation with a dose dependency indicates that <sup>166</sup>Ho-DTPA acts only through its own radioactivity and without any other toxicity. Additionally, the radioactivity of Ho-166 can be easily measured and predicted by dosimetry, which can be used to deliver the desired dose to cells. When <sup>166</sup>Ho-DTPA is used as a filling agent in a radioactive balloon, it presents advantages over the other proposed irradiation procedures, because Ho-166 as a  $\beta$ -emitter and unlike  $\gamma$ -emitters,

has limited tissue penetration and therefore, delivers a significantly lower dose to tissues beyond the target tissue. In solution,  $^{166}\text{Ho-DTPA}$  uniformly fills the expanded balloon making it possible for accurate source positioning, and for providing the most uniform and homogeneous dose to the vessel walls. In a case of balloon failure, the biological and radiological toxicity of Ho-166 may be circumvented by labeling with DTPA<sup>16,35</sup>. Therefore,  $^{166}\text{Ho-DTPA}$  can be used safely and conveniently for endovascular balloon brachytherapy as a mean of inhibiting coronary artery restenosis<sup>35</sup>.

VSMCs containing estrogen receptors specifically respond to estrogen<sup>42</sup>. Estrogen is a well-known inhibitor of VSMC proliferation and migration that play a major role in atherosclerotic vascular disease and in the remodeling process<sup>43</sup>. Estrogen also influences vascular function and structure, in a complex manner that involves numerous cellular and molecular mechanisms<sup>17</sup>. Most of the actions of estrogen appear to be exerted via the intracellular estrogen receptors, which are in turn members of a large superfamily of proteins that function as ligand-activated transcription factors, regulating the synthesis of specific RNAs and proteins<sup>44</sup>.

5% serum-stimulated RASMCs were treated with estrogen at the physiological concentrations to investigate cell proliferation. As shown in Figure 2, estrogen inhibited the cell proliferation in a dose-dependent manner up to 100 nM. Estrogen differentially inhibited the MAPK signaling in RASMCs. Anti-proliferative effect of estrogen in RASMCs was mediated by inhibition of p42/44<sup>ERK</sup>, but not p46/54<sup>JNK</sup> and p38<sup>MAPK</sup> MAPK subfamilies. Moreover, the inhibition of MEK1,2 caused the inactivation of p42/44<sup>ERK</sup> without the influences of the other upstream regulators, Ras and PKC. The induction of *c-myc*, *c-fos* and *elk-1* mRNA are some of the earliest

transcriptional events associated with cell proliferation. When evaluating the effects of estrogen on MEK1,2/p42/44<sup>ERK</sup> signaling and the subsequent suppression of proto-oncogene mRNAs, the inhibition of MEK1,2/p42/44<sup>ERK</sup> was found to cause a decrease in transcription of the *elk-1* gene in RASMCs. These results indicate that estrogen inhibits the activity of p42/44<sup>ERK</sup> and that this is followed by the suppression of MEK1,2, which leads to the down-regulation of *elk-1*.

Estrogen and  $\beta$ -emitting Ho-166 were effective at inhibiting the proliferation of RASMCs in a dose dependent pattern. To evaluate whether the anti-proliferative effect of estrogen and Ho-166 has a common subcellular mechanism, effect of low dose of Ho-166 in combination with estrogen was investigated upon ERK signaling which is the proliferation related MAPK. After RASMCs were treated with estrogen (40 nM) for 72 hours, the activity of ERK was measured at low Ho-166 dosage (0.3 Gy). Although Ho-166 inhibited RASMC proliferation effectively (Figure 1) and the increase of ERK activity in serum-stimulated RASMCs (Figure 6), it did not show a synergistic effect with estrogen on MAPK expression. This result indicates that the anti-proliferative mechanisms of Ho-166 and estrogen are distinct in RASMCs. As shown in the above results, estrogen has a dose-dependent effect on the cell proliferation and ERK activity in RASMCs. These indicate that the ERK signal cascade is the main mechanism of RASMC proliferation in the case of estrogen. However, though Ho-166 inhibited the increase of ERK activity in serum-stimulated RASMCs (Figure 6), this may indicate a nonspecific suppression of intracellular effector mechanisms including upstream regulators of MAPK, rather than a specific anti-proliferative mechanism of Ho-166. Recently, many investigators documented that  $\beta$ -emitting radiation interfered with cell cycle progression, by induction of G<sub>0</sub>/G<sub>1</sub>

arrest in VSMCs at low dosage, and inhibited VSMCs migration by 80% without any evidence of cell viability alteration or apoptosis<sup>45</sup>. In the case of  $\gamma$ -radiation, a portion of the effect on cell killing is mediated by the permanent interruption of the normal cell cycle progression of stimulated VSMCs<sup>46,47</sup>. Therefore, although radioactivities of <sup>166</sup>Ho-DTPA have diverse influences on subcellular mechanisms, the anti-proliferative effects of <sup>166</sup>Ho-DTPA may be predominantly associated with cell cycle interruption rather than MAPK inactivation. In terms of the interaction between radiation and MAPK, Takashi S et al reported that p38<sup>MAPK</sup> was activated by low dose X-rays as a radioadaptive response, but in high dosage, the down-regulation of labile PKC- $\alpha$  prevented p38<sup>MAPK</sup> activation<sup>48</sup>. In the case of this experiment, at above 1 Gy of radioactivity, MAPK activity could not be evaluated, which was probably caused by such down-regulation of upstream regulators. Even though the activity of the ERK signal was not suppressed in a synergistic manner by the estrogen and Ho-166 in combination, cell proliferation would nevertheless be inhibited more in combination than alone by the many other effector pathways of estrogen and radiation.

## V. Conclusion

$^{166}\text{Ho}$ -DTPA was highly effective at inhibiting the proliferation of RASMCs in a dose-dependent fashion, and therefore, a promising filling agent for intra-coronary balloon brachytherapy in the treatment of coronary artery restenosis. Estrogen also inhibited VSMC proliferation by selectively inhibiting ERK, a component of MAPK signal cascade, in a dose-dependent manner. The inactivation of ERK was selectively followed by the suppression of the activity of MEK1,2 as an upstream regulator and led to the suppression of the early gene *elk-1* transcription in a dose-dependent manner. Ho-166 did not show a synergistic effect on MAPK expression with estrogen, which indicates that the anti-proliferative mechanisms of Ho-166 and estrogen are distinct in RASMCs. Among the diverse influences on subcellular mechanisms of  $^{166}\text{Ho}$ -DTPA, the anti-proliferative effects of  $^{166}\text{Ho}$ -DTPA may be predominantly associated with cell cycle interruption rather than MAPK inactivation. Even though the activity of the ERK signal was not suppressed in a synergistic manner by the estrogen and Ho-166 in combination, cell proliferation would nevertheless be inhibited more in combination than alone by the many other effector pathways of estrogen and radiation.

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MAP Kinase

β-

<sup>166</sup>Ho-DTPA

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<sup>166</sup>Ho-DTPA 가 β-

dosimetry

hemocytometer

trypan blue dye-exclusion

MAP Kinase

western blot

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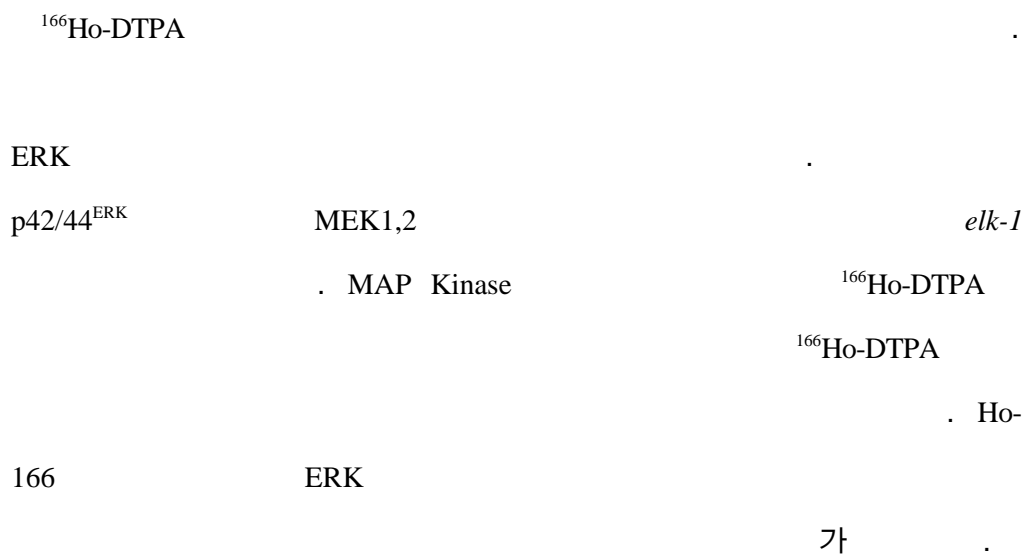
. *c-fos*, *c-jun*

*elk-1* proto-oncogene

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 Mitogen-activated protein kinases