行政院國家科學委員會補助專題研究計畫成果報告

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※ **The p38 Mitogen-Activated Protein Kinase Pathway** ※
※ **Regulates Left Ventricular Remodeling in Response to** ※
※ **Growth Hormone after Myocardial Infarction in Rats** ※
※ **p38 MAP Kinase 調節生長激素對左心室再塑造的角色** ※
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計畫類別：□個別型計畫□整合型計畫
計畫編號：NSC89－2314－B－002－186
執行期間：88年08月01日至89年07月31日

計畫主持人：蔡長和
共同主持人：李聰明

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- □國際合作研究計畫國外研究報告書一份

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行政院國家科學委員會專題研究計畫成果報告

The p38 Mitogen-Activated Protein Kinase Pathway Regulates Left Ventricular Remodeling in Response to Growth Hormone after Myocardial Infarction in Rats

p38 MAP Kinase 調節生長激素對左心室再塑造的角色

計畫編號：NSC89-2314-B-002-186

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一、中文摘要

心肌梗塞後，左心室發生的再塑造現象包括心肌死亡和尚存活心肌細胞的肥大。在細胞肥大的過程中，常會誘發一些胎兒基因的表現，如 β-myosin heavy chain (β-MHC)。適度的心肌肥大原是為維持心輸出量，但過度的肥大反而造成心臟功能的衰退。心肌梗塞早期若有給予生長激素會減緩左心室再塑造和改善心肌功能。然而真正的作用機轉尚不得而知。先前研究顯示生長激素對細胞的作用不單只來自 IGF-1 的分泌。對於 ERK 1/2 也有影響。但對於 p38 MAP Kinase 則從未被研究過。MAP Kinase 係細胞表面將訊號傳到細胞核的重要因子。目前被分離出的 MAP Kinase 超過 12 種，其中最重要的有三種：ERK, JNK 和 p38 每一種 Kinase 激活特定的細胞訊息。p38 MAP Kinase 可由發炎的反應或細胞壓力而激活，此種 Kinase 對基因調節，形態改變和細胞凋亡和細胞存活有密切關係。生長激素對於心肌梗塞後的左心室再塑造，機轉仍不得而知。本研究在探討生長激素在心肌梗塞後對左心室再塑造之影響，並探討其可能之機轉和 p38 MAP Kinase 之關係。在綁完左前降枝冠狀動脈後 ，大鼠被分成是否給予生長激素(2 IU/kg/day)四週。每組十隻。以心臟超音波檢查左心室再塑型和功能。與無心肌梗塞者做比較，可以清楚地看到大範圍之心肌梗塞會引起左心室擴大，心室功能下降。而生長激素則可減少左心室擴大之程度和改善心室功能。

結論：我們研究顯示心肌梗塞後若早期給予生長激素可使左心室有較佳之功能和減少左心室擴大之現象。

二、計畫成果摘要:(約二百字)

After myocardial infarction (MI), ventricular remodeling occurs which may be an adaptive process to the loss of myocytes, and consists of hypertrophy of the remaining cardiomyocytes. However, pathologic remodeling occurs in which dilation and resultant afterload excess combine to initiate deterioration in left ventricular function. Prevention or attenuation of this secondary process is an important therapeutic goal. Previous studies showed that early treatment of MI with exogenous growth hormone attenuates the pathologic left ventricular (LV) remodeling and improves left ventricular function in animals. The molecular mechanisms of growth hormone on LV remodeling after MI remain unknown. The present study was designed to explore the *in vivo* beneficial effects of growth hormone on the myocardium remodeling after MI and, if so, to determine whether the p38 MAP kinase is involved in expression of β-MHC induced by growth hormone. Following ligation of the left coronary artery or sham operation, rats were randomized to receive 2 IU/kg/day or vehicle for four weeks (n = 10 in each group). LV remodeling and function were assessed by echocardiography. Compared to sham, vehicle-treated rats with large (>20%) infarct size showed LV dilation, reduced fractional shortening well as

LV/body weight, and LV posterior wall thickness. Treatment with growth hormone attenuated the LV dilation and improved LV function.

Conclusions.
Our study demonstrated that growth hormone, given early after large MI, elicits benefits of structural effects characterized by preserved LV function and attenuated LV dilation.

關鍵詞: β-myosin heavy chain, Growth hormone, Intracardiac ultrasound, Myocardial infarction, Northern blot, p38 MAP kinase, Ventricular remodeling, Western blot.

三、計畫簡介（Introduction）

After myocardial infarction (MI), a massive myocardial necrosis and a reconstructing process called "ventricular remodeling" occur. This ventricular remodeling may be an adaptive process to the loss of myocytes, and consists of hypertrophy of the remaining cardiocytes. During the hypertrophic response, cardiomyocytes activate a distinct pattern of gene expression that eventually results in qualitative and quantitative alterations in contractile protein content (e.g. ventricular myosin light chain-2, cardiac muscle α-chain) and the induction of a fetal gene program such as β-myosin heavy chain, atrial natriuretic factor, and brain natriuretic factor (1). This reactive hypertrophy assists in maintaining the cardiac output. However, pathologic remodeling occurs in which dilation and resultant afterload excess combine to initiate deterioration in left ventricular function. Prevention or attenuation of these secondary process is an important therapeutic goal (2).

Recently, studies showed that early treatment of MI with exogenous growth hormone (GH) attenuates the pathologic left ventricular remodeling and improves left ventricular function in animals (3,4). Penney et al (5) also showed in rats that implantation of GH-secreting tumors significantly improved myocardial contractility and cardiac output. Increasing evidences have demonstrated that GH provides beneficial effects on cardiac remodeling under experimental and clinical conditions. However, the precise mechanisms by which GH induces the signaling machinery remain unclear. This gonodal steroid hormone exerts most of their effect by direct binding to specific nuclear receptors, which act as transcription factors (6). It is recognized that GH effects on cell function are not mediated solely via the promotion of insulin-like growth factor secretion from the liver (7). Previous studies showed that phosphorylation of extracellular-signal-regulated kinases (ERK)-1/2 mitogen-activated protein (MAP) kinases on tyrosine and threonine residues is responsible for the action of GH (8,9). However, the effects of GH on p38 MAP kinases have not previously reported.

The MAP kinases are important components in signal transduction from the cell surface to the nucleus. Over a dozen MAP kinases have been cloned and the best characterized of these are the ERK, the c-Jun N-terminal kinase (JNK) and p38 (10). Each MAP kinase is activated by dual phosphorylation of a conserved threonine and tyrosine residue in the activation loop. However, each kinase activates a unique spectrum of cellular targets, and defines independent signaling pathways, which are responsive to different extracellular stimuli. The identification of distinct MAP kinase kinases with specifically activate ERK, JNK or p38 MAP kinases suggest the existence of independent signalling roles for these MAP kinase cascades (11). The p38 MAP kinase activity is activated by inflammatory cytokines, and cellular stresses such as heat shock, osmotic stress or ultraviolet light (12,13). p38 MAP kinases have been implicated in gene regulation, morphological alteration, apoptosis and cell survival in response to various environmental stimuli (14-16).

The role of MAP kinase in left ventricular remodeling remained uncertain.

Intracellular molecular mechanisms of cardiac adaptation have been examined exclusively *in vivo* and *in vitro* remodeling responses. It is likely that some of the cardiac adaptation involve modulation of transcription factor activity in which the MAP kinase participates. Sadoshima et al (17) showed in a model of cultured cardiac myocytes that multiple signaling pathways such as MAP kinase cascade were activated in response to hypertrophy. MAP kinase seemed to play an important role in myocardial hypertrophic processes, probably by acceleration protein synthesis and regulating the function of the transcriptional factors. p38 kinase activities are significantly induced in transgenic mouse hearts expressing activated Ha-Ras, correlating with the onset of cardiac hypertrophy (18). Recently, Wang et al showed that p38 MAP kinase activities are elevated in associated with the onset of hypertrophy and apoptosis in a model of chronic aortic constriction (19). MAP kinase positively regulated the activity of the specific promoter regions such as AP-1 site and serum response element by phosphorylating *c-Jun* (20). These data suggest that the activation of MAP kinase cascade was early intracellular events in response to growth stimuli in cardiac myocytes.

The molecular mechanisms of GH on left ventricular remodeling after MI remain unknown. The present study was designed to explore the *in vivo* beneficial effects of GH on the myocardium remodeling after MI and, if so, to determine whether the p38 MAP kinase is involved in expression of β-MHC induced by GH.

四、材料及方法(Subjects and Methods)

Animals.

Procedures for animal care, surgery, and euthanasia were approved by our institutional review committee for animal experiments. Male normotensive Wistar rats that weighed 150-200 g fed a normal sodium diet, with a sodium content of 0.32 wt% and offered tap water ad libitum before the study. Fifty anesthetized rats were subjected to one of five protocols (10 each group). On the same postoperative day (D0), after the echocardiogram, the animals were randomly assigned to receive drugs (GH, SB203580) or placebo for 4 weeks. Group A (Sham) was the control group and sham operation was performed. In Group B (MI) animals received coronary ligation without drug intervention. In Group C (MI plus GH) animals received coronary ligation and GH administration from D1. In Group D (MI plus SB) animals received coronary ligation and SB203580 from D1. In Group E (MI, GH and SB) animals received coronary ligation and administered GH and SB203580 from D1. The study duration was designed to be 4 weeks because the majority of the myocardial remodeling process in the rat (70-80%) is complete within 3 weeks (21). GH was administered at the dose of 2 IU/kg/day, which was suggested by Grimm et al (4). SB203580 was dissolved in drinking water, and the concentration (expected serum level about 10 μM) was adjusted for the daily water intake and body weight to obtain an average daily dose of 0.4 mg/kg body weight. SB203580 specifically inhibited p38 MAP kinase with an IC_{50} of 0.6 μM, and even at 100 μM had no effect on the activities of 12 other protein kinases, including ERK and JNK (10). Moreover, SB203580 prevented the activation and phosphorylation of MAPKAP kinase-2 and the phosphorylation of hsp27 by cellular stresses (2,22). They were kept in cages, 6 per cage, in a standard light/dark room at a constant temperature (22 ± 1^{0}C) and humidity.

Experimental MI.

Rats were intubated under general anaesthesia (thiopental 50 mg/kg, i.p.) and placed on a respirator. The heart was exposed via a left-sided thoracotomy, and the anterior descending artery was ligated between the pulmonary outflow tract and the left atrium. Sham rats underwent the same procedure except the suture was passed under the coronary artery and then removed.

Intracardiac Echocardiographic

examination

Animals received an intracardiac ultrasound (ICUS) examination at the operation day and the 28th day after the operation (D28) as previously described (23). Briefly, a commercially available ICUS system (CVIS, Sunnyvale, CA) with a 10-Fr 10-MHz catheter was used. To facilitate apposition of the catheter with the esophagus, the tip of the catheter was preshaped with a 0.018-inch diameter guide wire to produce a gentle curvature of the distal portion of the catheter. After lubrication with lidocaine gel, the catheter was carefully inserted into the esophagus. A short-axis view of the left ventricle at the midpapillary muscle level was obtained for analysis (**Figure 1**). The length that the catheter was inserted was recorded for future repeat examination. Heart rate was determined from a continuous electrocardiographic tracing. Baseline heart rate and systemic arterial pressure were measured. Echocardiographic left ventricular mass was determined using the formula proposed by De Simone (24):

Left ventricular mass (in mg) = 1.05 x (ED^3_{ed}-ID^3_{ed}), where 1.05 is the specific gravity of muscle, and ED_{ed} is the external dimension of the left ventricle at end diastole, and ID_{ed} is the internal dimension of the left ventricle at end diastole, both measured in millimeters.

Left ventricular fraction shortening (%)=[(ID_{ed} –ID_{es} / ID_{ed}]*100, where ID_{es} is the internal dimension of the left ventricle at end systole measured in millimeters.

Blood pressure measurements

Functional parameters were measured in anesthetized rats. Systolic blood pressure was measured by means of the tail cuff technique using the Harvard apparatus system. Each value is the average of three consistent readings. After 28 days, the rats were anaesthetized with thiopental sodium (50 mg/kg, ip) prior to performing hemodynamic and echocardiographic measurements.

Infarct size measurements

Infarct size was determined by a previously described technique (25). The left ventricle was embedded in paraffin, and 20 μm sections were cut serially from apex to base of the heart. Sections were stained with Picrocirius Red and were mounted on glass plates, scanned and planimetry was performed with Image Pro Plus software. Lengths of scar and noninfarcted muscle for both endocardial and epicardial surfaces were determined each section. Final infarct size was determined as the average of endocardial and epicardial surfaces and is given in percent. With respect to clinical importance, only rats with large MI were selected for detailed investigation.

After the above procedure, the left ventricle was infused with 50 ml diethyl pyrocarbonate (Sigma)/PBS to wash blood cells and to avoid RNA degradation. The left ventricle with septum and right ventricle was separated and frozen rapidly in liquid nitrogen, and stored at –80^{0}C until use for RNA analysis.

Northern Blot Analysis

Because the effects of GH appear to be mediated in part by inhibition of β-MHC expression (26), we measured the ventricular level of β-MHC mRNA by Northern bloting. Each frozen tissue sample was first pulverized and then RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform method as described by Chromczynski and Sacchi (27). The RNA concentration was determined spectrophotometrically by absorbance at 260 nm. For each sample the total RNA (15 μg) was denatured in formaldehyde, run in 1.2% agarose-formaldehyde gel and transfer overnight onto a positively charged nitrocellulose membrane (Gene screen plus; Dupont, Wilmington, DE). The transfer was done by an adsorption process using 10 x SSPE(1.5M NaCl, 0.1M $NaH_2PO_4xH_2O$, 0.01M EDTA) as a transferring buffer. The filters was incubated in prehybridization buffer (5 x SSPE, 50% formamide, 5 x Denhardt's solution, 1% SDS, 100 g/ml denatured salmon sperm DNA [Pharmacia

Fine Chemicals. Piscataway, NJ]) for 4 h at 42 ℃. The filters was then hybridized overnight at 42℃ in fresh prehybridization buffer containing the denatured ^{32}P-labeled rat β-MHC-specific probe (sp.act .2× 10^7 cpm/g). The steady state expression of β-MHC was quantitated by scanning densitometry (Zeineth Soft Scanning Densitometer model SLR-2D/ID-DNA; Biomed. Instruments Inc., Fullerton, CA). Experiments were replicated three times.

To confirm equality of loading of the extracted RNA from the heart samples, the same blots were further washed with 0.1× SSPE and 0.1% SDS at the boiling temperature for 15 min and rehybridized with rat GAPDH and β-actin probes, which are housekeeping genes to act as control. The hybridization signals of specific mRNAs were divided by those of GAPDH mRNA to correct for differences in RNA loading and/or transfer.

Western Blot Analysis

Left ventricles were homogenized in extracting buffer (10 mM Tris, pH .4, 1.0 mM phenylmethysulfonyl fluoride, 10 μg/ml of leupeptin, and 0.1% Triton X-100). Protein concentration was determined with the BCA protein assay reagent kit (Pierce). Twenty-five μg protein was separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane. After incubation with rat monoclonal antibodies generated to a peptide containing rat β-MHC, the nitrocellulose membrane was then rinsed in Blottto solution and incubate for 2 hours at room temperature. β-MHC protein was detected using a 1:1000 dilution of β-MHC monoclonal antibody (Chemicon) and an enhanced chemiluminescence kit (ECL, Amersham) according to the manufacture's protocol. A horseradish peroxidase-linked secondary anti-mouse antibody (Amersham) was used at a final dilution of 1:2500, and the film was exposed for 45 minutes. Autoradiographic bands of films were volume-integrated within the linear range of the exposure using a scanning densitometer. Experiments were replicated three times.

In Vitro MAP kinase assays.

Protein extracts from frozen heart were prepared and assayed for kinase activities as described in the Section of Western Blot Analysis. P38 kinases were immunoprecipitated using rabbit polyclonal anti-p38 antiserum (Scripps, Research Institute, La Jolla, CA and Santa Cruz Biotechnology) conjugated to protein A-Sepharose. The kinase assays were then performed at 30^{0}C using [γ-^{32}P]ATP and myelin basic protein (Sigma) as a substrate. The phosphorylated substrate was separated by 12% acrylamide SDS-polyacrylamide gels and stain with Coomassie Blue. The gels were dried and radiograph at –70°C.

Statstical Analysis

Results were presented as mean ± SD. Differences among the groups of rats were tested by a one-way ANOVA. Subsequently analysis for significant differences between the two groups was performed with a multiple comparison test (Scheffe's method). The correlation between continuously distributed variables was tested by univariate regression analysis. The significant level was assumed at value of $P<0.05$.

五、結果(Results)

Hemodynamics.

Because the specific antagonist of P^{38} MAP kinase, SB203580, was not available from SB Company, we changed our protocol to as follows. We randomized the rats into four groups. Group 1 (*Sham*): rats underwent the same operation, but did not receive the ligation of the coronary artery. Group 2 (*MI*): animals received the ligation of the proximal left anterior descending artery. Group 3 (*GH*): for comparison of effects of GH, the rats undergone sham operation received GH at the dose of 2 IU/kg/day through subcutaneous injections. Group 4 (*MI + GH*): the rats undergone MI operation received GH at the dose of 2 IU/kg/day through subcutaneous injections. A representative MI is shown in Figure 2.

Perioperative mortality.

According to previous reports (3) and our experience, the perioperative mortality within the first 48 hours following ligation of the left anterior descending artery is high up to 80%, irrespective of whether the animal was treated with GH.

Hemodynamics.

There were no significant changes in blood pressure and heart rate among the groups (Table 1).

Somatic and cardiac weights, and echocardiography.

Compared with the sham group, the MI group showed LV dilation and increased posterior wall thickness (Table 1). Rats with MI had a significantly lower BW compared to sham-operated rats, and treatment with GH attenuated these findings. MI was associated with an increase in LV mass, which was exaggerated following treatment with GH, in particular in the case of a large MI. LV dilation, as assessed by *in vivo* echocardiography, occurred in all rats with MI. GH treatment significantly reduced the LV size but increased mass. LV fractional shortening as a measure of contractility significantly improved subsequent treatment with GH.

六、討論(Discussion)

This study demonstrated several beneficial effects of GH treatment on the remodeling process after MI. There was a reduction of LV dilation and improvement of systolic function. Hypertrophy of the noninfarcted myocardium with GH was documented by echocardiographic assessment. Previous studies have demonstrated that IGF-1, given in the early phase of postinfarction remodeling in the rat, induced a hypertrophic response and increased systolic function (28). Our finding was consistent with the previous study.

Clinical Implications

Considering that LV dysfunction due to myocardial infarction is the leading cause of congestive heart failure, the beneficial effects of GH may have an important clinical impact. Therapeutical options in patients with MI include the administration of ACE inhibitors and β-adrenoceptor blockers. Their use ameliorates pathological remodeling, improves symptoms and prolongs survival. GH, with its distinct effects on cardiomyocyte remodeling, might become a means of further improving cardiac function.

七、自評

由於無法向 SB 公司取得 P38 MAP Kinase specific antagonist: SB203580，因此使本實驗只能算是完成一半，我們仍將繼續努力向 SB 公司爭取以完成整個實驗。另外 Growth Hormone 單價過高，每完成一隻 Rat，幾乎要花 4~5 萬元(因為失敗率約五成)，使經費捉襟見肘。

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Table 1. Growth hormone in experimental myocardial infarction.

Parameters	*Sham (n =10)*	*MI (n = 10)*	*GH (n =10)*	*MI+GH (n = 10)*
Heart rate (bpm)	356 ± 27	325 ± 18	319 ± 22	335 ± 25
Blood pressure (mm Hg)	112 ± 13	107 ± 16	118 ± 14	105 ± 11
BW (gram)	385 ± 26	342 ± 25*	398 ± 21	378 ± 19
LV/HW	0.67 ± 0.05	0.73 ± 0.01*	0.74 ± 0.03	0.78 ± 0.04†
LVEDD (mm)	4.8 ± 0.5	5.4 ± 0.4*	4.6 ± 0.5	5.1 ± 0.5
LV mass (g)	0.82 ± 0.13	1.03 ± 0.16	0.98 ± 0.21	1.29 ± 0.20†
FS (%)	67 ± 5	54 ± 6*	72 ± 7	66 ± 5†

BW, body weight; FS, fractional shortening; GH, growth hormone; HW, heart weight; LV, left ventricle, LVEDD, left ventricular end-diastolic dimension (mm); MI, myocardial infarction. * $P<0.05$ compared with the sham group; †$P<0.05$ compared with the MI group.

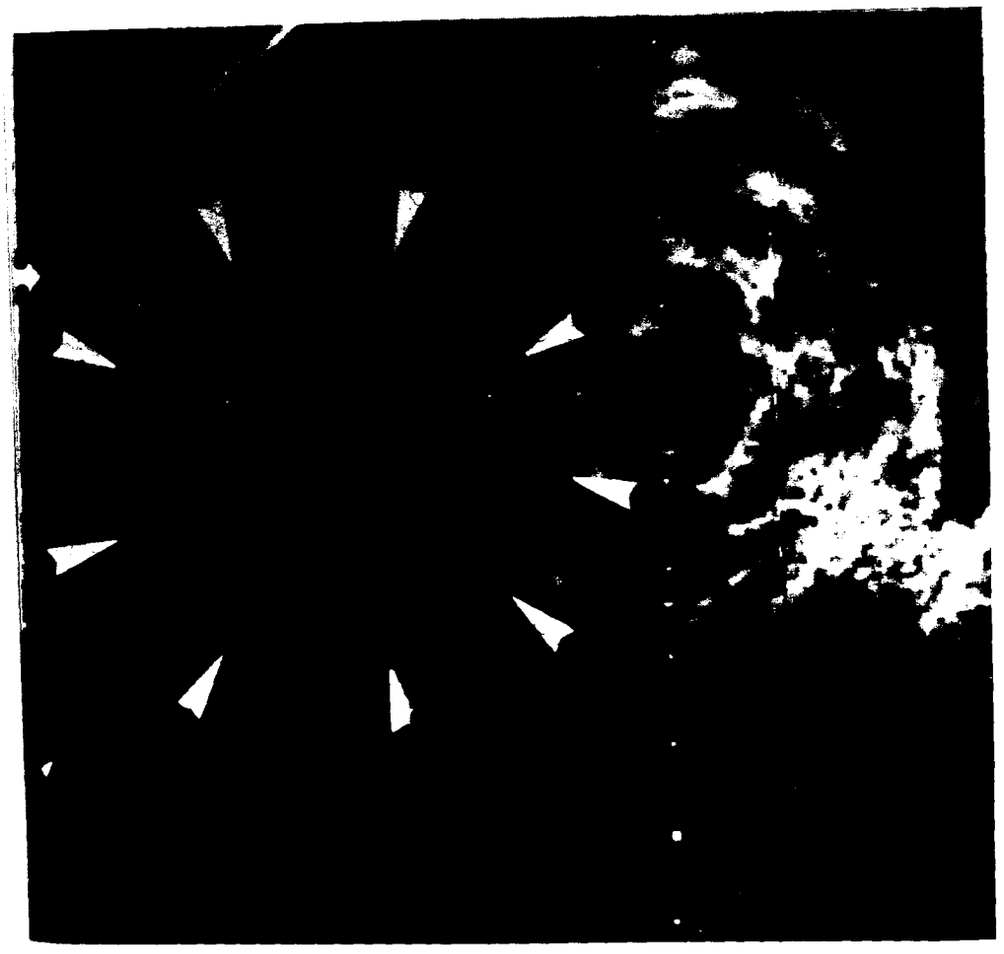

Figure 1. Short-axis view of the left ventricle at midpapillary muscle level with a 10-Fr, 10-MHz intracardiac ultrasound catheter.

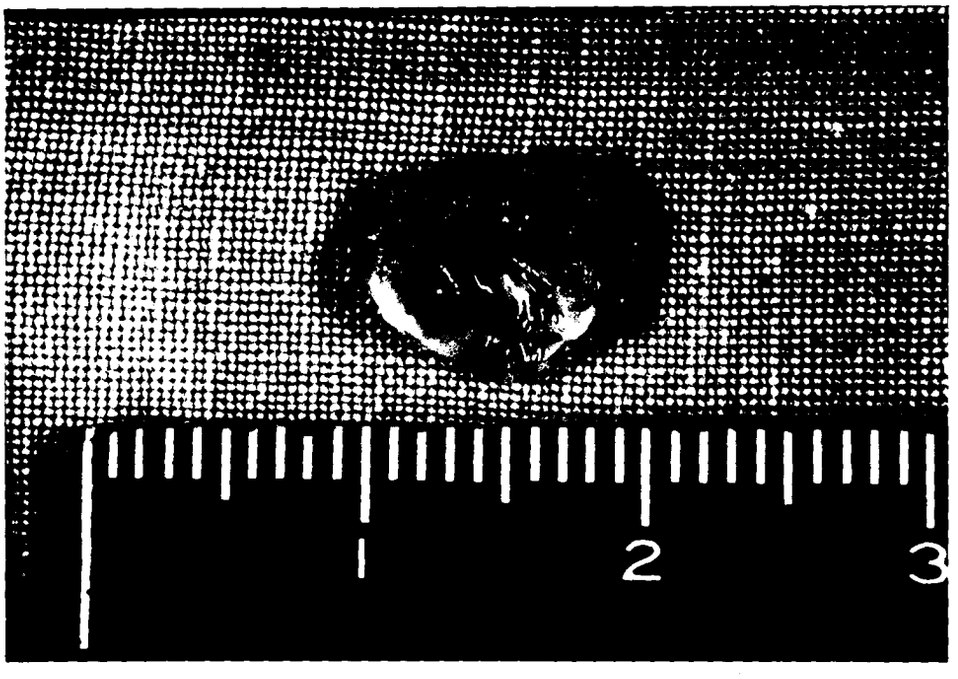

Figure 2. A representative myocardial infarct is shown in the group treated with growth hormone.