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Targeted Delivery of Vascular Endothelial Growth Factor Improves Stem Cell Therapy in a Rat Myocardial Infarction Model

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Abstract

Rebuilding of infarcted myocardium by mesenchymal stem cells (MSCs) has not been successful because of poor cell survival due in part to insufficient blood supply after myocardial infarction (MI). We hypothesize that targeted delivery of vascular endothelial growth factor (VEGF) to MI can help regenerate vasculature in support of MSC therapy in a rat model of MI.

VEGF-encapsulated immunoliposomes targeting overexpressed P-selectin in MI tissue were infused by tail vein immediately after MI. One week later, MSCs were injected intramyocardially. The cardiac function loss was moderated slightly by targeted delivery of VEGF or MSC treatment, while targeted VEGF + MSC combination treatment showed highest attenuation in cardiac function loss. The combination treatment also markedly increased blood vessel density (80%) and

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decreased the collagen content in post-MI tissue (33%). Engraftment of MSCs in the combination treatment group was significantly increased and the engrafted cells contributed to the restoration of blood vessels.

Keywords

angiogenesis; myocardial infarction animal model; liposome; mesenchymal stem cells; myocardial infarction

Introduction

Myocardial infarction (MI) is the leading cause of morbidity and mortality in most countries¹. MI occurs when blood supply to the heart is disrupted for a period long enough to result in cell loss and myocardium necrosis. The damaged area is eventually replaced by scar tissue to maintain structural integrity. Current clinical treatment procedures focus first on restoring the antegrade flows and minimizing pathologic remodeling of the MI. Heart function can then be improved by administration of inotropic drugs to boost the mechanical performance of the remaining cardiomyocytes². Although these treatment modalities may ameliorate symptoms, they do not completely recover the function of the damaged tissue because the regeneration capability of myocardium is very limited³.

In order to restore heart function, MI area needs to be rebuilt by generating new myocytes. Tissue engineering approaches employing stem cells have achieved laboratory success in this respect as stem cells can be induced to differentiate into cardiac myocytes and thus have the potential to regenerate new myocardium^{4, 5}. However, attempts at rebuilding injured tissue *in vivo* using transplanted stem cells have yet to be successful due in part to a lack of blood supply to the MI region leading to poor viability and increased apoptosis of the transplanted cells⁶. Pro-angiogenic compounds, such as vascular endothelial growth factor (VEGF), have been shown to stimulate blood vessel re-growth within the MI area^{7, 8}. However, systemic delivery of VEGF has very limited therapeutic effects⁹ and can produce many adverse side effects such as the development of neoplasms, diabetic retinopathy, rheumatoid arthritis, and atherosclerosis^{6, 10}. Targeted delivery strategies can concentrate VEGF at the MI site thus maximizing its therapeutic potentials while minimizing its side effects. Previously, we have reported that targeted delivery of VEGF to infarcted myocardium by anti-P-selectin conjugated immunoliposomes results in a significant increase in the number of both anatomical and perfused vessels in the MI region in rats and improves cardiac function⁹. In the current study, we show that targeted delivery of VEGF to MI can stimulate regeneration of vasculature, and, in combination with transplantation of MSCs, can further significantly improve cardiac function after MI in a rat model.

Materials & Methods

A. Rat MI Model

Surgical MI was induced in 6 week old male Sprague Dawley rats (Charles River Laboratories International, Inc, Wilmington, MA, USA) as described previously¹¹. Briefly, rats were anesthetized with isoflurane, intubated and ventilated with a rodent ventilator (Euthanex Corporation, Allentown, PA, USA). The left anterior descending coronary artery was occluded with silk ligature. Evidence of MI was confirmed by S-T segment elevation and the appearance of Q wave on an electrocardiogram. After surgery, rats were randomly assigned into five experimental groups: I) Sham operated (rat chest opened/closed without ligation), n = 5 rats; II) MI but untreated (saline injection), n = 5 rats; III) targeted delivery of VEGF treatment, n = 7 rats; IV) MSC treatment, n = 7 rats; and V) targeted delivery of VEGF + MSC combination treatment, n = 10 rats. Systemic delivery of VEGF was not included in the current study since we have previously shown that it does not result in significant improvements in cardiac function¹². All animal handling protocols were approved by the Temple University Institutional Animal Care and Use Committee. Buprenorphine was given to the animals before surgery to prevent residual pain during surgery and after surgery to relieve pain and stress during the recover from anesthesia. The animals were then placed on a heating pad and observed every 15 minutes until fully recovered from anesthesia as indicated by the ability to maintain sternal recumbency and moving normally, at which point they were moved back to the cage. After recovering from anesthesia, the animals were observed every 30 minutes for the first 8 hours. After 8 hours, they were checked daily until 7 days post-op and once every other day thereafter for 3 more weeks. Animals that appeared to be in distress or pain (muscle spasm, loss of balance, loss of weight, etc.) during the 4 week observation period were euthanized using an overdose of KCl (2 meq/kg) injected via the tail vein under gas anesthesia.

B. MSC Culture

Rat bone marrow-derived MSCs expressing EGFP (Enhanced Green Fluorescent Protein) were obtained and cultured as described before¹³. Briefly, adult Sprague Dawley rats were euthanized, femurs and tibias were removed and bone marrow was flushed out under sterile conditions and plated in α -MEM (Mediatech, Inc., Herndon, VA, USA) supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA, USA), 2 mM L-glutamine, 100 μ g/mL penicillin/streptomycin, and 25 μ g/mL amphotericin B (Mediatech, Inc., Herndon, VA, USA). The MSCs were isolated from the hematopoietic component of the bone marrow by their adherence to tissue culture plastic. The non-adherent cells were discarded after 48 hours and the adherent cells were washed twice with phosphate-buffered saline (PBS) and the media replaced. After the cells grew to near confluency, the cultures were harvested with trypsin (0.25%) and, MSCs were expanded further by plating at about 5,000 cells per cm² in α -MEM media supplemented with 10% FBS, 10 ng/ml of basic Fibroblast Growth Factor (Invitrogen, Carlsbad, California, USA) and 10 ng/ml of Epidermal Growth Factor (Invitrogen, Carlsbad, California, USA). The cells were then labeled with EGFP and cultured in the same conditions for future applications. Multipotent characteristics of MSCs were tested through *in vivo* experiments as well as using standard *in*

vitro assays designated to test differentiation along osteogenic and adipogenic lineages¹³. MSCs were obtained under a protocol approved by the institutional IACUC.

C. Preparation of Immunoliposomes

The targeted drug delivery system was produced by a two-step process as described previously¹⁴. First, VEGF (Recombinant Human Vascular Endothelial Cell Growth Factor-165, Genentech, San Francisco, CA, USA) encapsulated long circulating liposomes, composed of 50% hydrogenated soy L- α -phosphatidylcholine (HSPC), 45% cholesterol, 3% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[(polyethylene glycol)2000] (DSPE-PEG2000), and 2% DSPE-PEG-maleimide, were prepared by means of solvent evaporation and film formation. All lipids were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). Second, anti-P-selectin monoclonal antibody (courtesy of Dr. Andrew Issekutz) was attached to the distal end of PEG chains on the liposome surface to form immunoliposomes. P-selectin was chosen as the target receptor because it is upregulated on the vasculature of the infarct tissue¹⁴. The antibody was first thiolated with 2-iminothiolane (Sigma-Aldrich Corporation, St. Louis, MO, USA) at pH 8.0. The introduced thiol groups were then coupled with maleimide groups on DSPE-PEG2000 component of the liposomes at pH 6.5. Unconjugated antibodies were removed by HiTrap heparin column (GE Healthcare Biosciences). Previously¹⁹ we have shown that this targeted drug delivery system can successfully and selectively deliver encapsulated drug to the MI tissue.

D. Administration of VEGF & MSCs into rat MI model

Immunoliposomes containing VEGF (1 mL of liposome/kg of animal weight @ 10 mM lipid concentration corresponding to 200 ng of VEGF/kg of animal weight) were administered via the tail vein immediately after induction of MI. previously we have shown that targeted delivery of VEGF at this concentration significantly increases the number of anatomical and perfused vessels after MI⁹. MSCs at passage 2 were injected intramyocardially one week post-MI induction. Briefly, a syringe was used to inject a total of 100 μ L of MSCs in saline at a concentration of 10,000 cells/ μ L equally to 4 different sites (25 μ L at each site) around the MI area in order to achieve a homogenous stem cell distribution. Accordingly, the saline group received saline treatments at both the time of the MI induction and 1 week post-MI; The VEGF only group received immunoliposomes containing VEGF at the time of MI induction and saline injection at 1 week post-MI; The MSC only group received saline treatment at the time of MI induction and MSC injection at 1 week post-MI.

E. Histology & Immunohistochemistry (IHC)

Four weeks after MI surgery, animals were sacrificed, and hearts were removed and ventricles were filled with OCT (Fisher Scientific) to prevent chamber collapse. The hearts were then flash frozen in a dry ice/2-methylbutane (Fisher Scientific Co., Houston, TX, USA) bath and stored at -80°C . Prior to staining, hearts were first sectioned at 6 μ m slices at -20°C using a cryostat (Leica CM3050 S, Buffalo Grove, IL, USA) and then mounted on poly-L-lysine-coated glass slides (Superfrost plus, Fisher Scientific Co., Houston, TX, USA) for staining and imaging.

The extent of tissue remodeling in the MI area was visualized using Gomori's Trichrome and Picrosirius red staining (both from Polysciences, Inc., Warrington, PA, USA). Anatomical blood vessels were identified by immunohistochemical staining for CD31 protein (BD Biosciences, Franklin Lakes, NJ, USA). The number of identified blood vessels was then counted by a built-in function in ImagePro software (Media Cybernetics, Bethesda, MD, USA). Engrafted EGFP expressing MSCs were identified by four different immunostaining techniques: monoclonal anti-cardiac troponin T antibody and monoclonal anti- α -actinin antibody were used to label cardiomyocytes; monoclonal anti- α -smooth muscle actin together with monoclonal anti-vimentin were used to identify newly formed blood vessels^{15–17} (Thermo Fisher Scientific Inc., Rockford, IL, USA).

F. Image Processing

A TE200 inverted microscope (Nikon Instruments, Inc., Melville, NY, USA) was used to obtain images from stained sections. Images of a whole heart section were obtained from a mosaic of individual images taken with the aid of a LEP MAC 5000 motorized stage (Ludl Electronic Products Ltd., Hawthorne, NY, USA) controlled by ImagePro. Anti-cardiac troponin T, anti- α -actinin, anti- α -smooth muscle actin and anti-vimentin staining were imaged using their corresponding fluorescence channels. These images were then superimposed with MSC fluorescence images to determine the viability and differentiation of individual stem cell. CD31 images were taken under bright field and enhanced using ImagePro to identify AEC-stained blood vessels. For each heart section, the number of vessels in the normal area as well as infarct area were quantified and compared.

G. Data Analysis

Rat heart function was monitored by echocardiography at 1 week and 4 weeks post-MI. Left ventricular percent fractional shortening (FS) data were used to determine the heart function. Left ventricle end diastolic dimension (LVEDD, mm) and left ventricle end systolic dimension (LVESD, mm) were measured directly by echocardiography and FS was calculated by:

$$FS = \frac{LVEDD - LVESD}{LVEDD} * 100\%$$

Since normal rats usually have a FS value of greater than 40%, rats with a FS value of less than 30% at 1 week post-MI were considered having a fully developed MI. A one-sided paired *t*-test was performed for each treatment between 1 week and 4 weeks post-MI. Differences among the groups at the 4 weeks post-MI were determined using one way ANOVA with SNK post-hoc correction. Data are presented as group mean $\pm$ standard error of the mean.

Results

A. Fractional Shortening Changes

Left Ventricle Fractional Shortening (FS) is a standard measure of the pumping function of the heart. There is no change detected (by paired *t*-test) in sham operated rats between the

first and the fourth week post-MI (FS: $44.6\% \pm 4.6\%$ to $43.7\% \pm 6.9\%$). However, there was a significant loss in cardiac function in the untreated group from first to fourth week post-MI (FS: $18.7\% \pm 2.7\%$ to $10.4\% \pm 3.4\%$, $p < 0.001$ by paired t-test). Either VEGF only treatment or MSC only treatment significantly attenuated the loss of cardiac function from first to fourth week post-MI (FS in VEGF group: $21.0\% \pm 5.2\%$ to $17.2\% \pm 4.8$, $p < 0.01$; FS in MSC group: $20.1\% \pm 2.9\%$ to $16.8\% \pm 5.0\%$, $p < 0.01$). The VEGF + MSC combination treatment group showed the highest attenuation in the loss of cardiac function after MI ($17.5\% \pm 2.9\%$ to $16.8\% \pm 4.5\%$, not significantly different). As shown in Figure 1, the FS difference between 1 week post-MI and 4 weeks post-MI (FS loss (%) in Y-axis) in the VEGF + MSC combination treatment group was significantly lower than in the control group and the group treated with MSC or VEGF ($p < 0.001$ by ANOVA). Independent histological examination by Dr. Stanley D Kosanke from the University of Oklahoma Health Sciences Center confirmed that there was no inflammatory response in major organs after targeted delivery of immunoliposomes.

B. Morphological Change & Tissue Remodeling in MI Area

Picrosirius Red (Figure 2) staining was used to visualize collagen content in the MI area for non- treated and treated heart sections. When Sirius red in saturated, picric acid selectively binds to fibrillar collagens (types I to V) and enhances the birefringence of fibrillar collagen. The specificity of Sirius red staining for fibrillar collagen enables sensitive quantitative measurements of collagen content to be performed in locally defined tissue areas. Following our previous work¹⁸, our quantification of collagen was based on the average intensity of red in the MI area. Although the size of the infarct varies in each sample, quantitative analysis suggests that the percentage of collagen in the MI area decreased significantly with different treatments. As shown in Figure 3, as compared to untreated MI samples, the collagen content decreased by 16%, 24%, and 33% for MSC only, VEGF only, and VEGF + MSC combination treatment, respectively. The VEGF + MSC combination treatment showed the highest reduction of fibrosis in the MI tissue, indicating less scar formation after the combination treatment.

C. Blood Vessel Regeneration in MI Area

Anatomical blood vessel density was quantified by CD31 staining. Due to the fact that anatomical vessel density varies in different hearts, but stays the same in the same heart¹¹, we normalized the vessel density by calculating the ratio of vessel density in the MI region of each heart sample to that in the non-MI region of the same sample (Figure 4). As shown in Figure 4, at 4 weeks post-MI, blood vessel density of the untreated MI group decreased significantly while the remaining blood vessels appear to have larger diameters (upper panel). Improvements in heart function by VEGF only treatment or the VEGF + MSC combination treatment were accompanied by a marked increase in vascular density (Figure 4, lower panel). Compared to untreated MI group, transplantation of MSCs alone resulted in a slight but not significant increase in the number of anatomical blood vessels. The VEGF only treatment significantly promoted angiogenesis in the MI area, which is consistent with our previous findings⁹. The VEGF + MSC combination treatment resulted in further increase in angiogenesis in the MI area as indicated by the highest number of anatomical blood vessels in MI area.

D. MSC Engraftment

The total number and density of EGFP expressing MSCs were determined from their fluorescence signature by measuring the area and intensity of green fluorescence using the ImagePro software. As shown in Figure 5, VEGF only treatment greatly increased the total number and density of engrafted MSCs. As shown in Table 1, quantitative analysis indicated a 49.8% increase in total engrafted MSCs and 58.4% higher MSC density in VEGF + MSC treated MI group than in the MSC treated group.

E. Fate of MSCs

The phenotype of MSCs was determined by immunohistochemistry three weeks after cell transplantations. As shown in Figure 6A and 6B, in the MI region, both anti-cardiac troponin T and anti- α -actinin immunoreactivities were weak in EGFP positive cells indicating that only a small portion of the implanted stem cells differentiated into cardiac myocytes in the MI area 3 weeks after the transplantation. However, most of the cells in the MI region positively stained for vimentin and anti-smooth muscle actin, Figure 6C and 6D, indicating that the majority of the implanted stem cells contributed to the formation of blood vessels after MI.

Discussion

In this study, we observed that the combination therapy using targeted VEGF delivery and MSC transplantation to MI area in a rat model resulted in the most significant improvement in cardiac function as measured by the change in heart fractional shortening 4 weeks post-MI as compared to either VEGF only treatment or MSC only treatment. Our data shows that targeted delivery of VEGF to the MI area greatly improves angiogenesis after MI and suggests that the local microenvironment, preconditioned by targeted delivery of VEGF treatment, correlates with the engraftment of stem cells in the MI area and more robust formation of blood vessels after MI. However, despite the significant improvements in cardiac function, we observed that very few stem cells underwent cardiac myocyte differentiation which may have limited the overall treatment efficacy.

Combining VEGF therapy with MSC transplantation has been performed previously. Several studies, which have featured a simultaneous treatment protocol, either by co-injection of stem cell and VEGF¹⁹ or transplanting gene transferred stem cells that overexpress VEGF^{20, 21}, have shown some promising results. In order to minimize the side effects of systemic delivery of VEGF, while extending the residence time of VEGF in the MI area, several targeting strategies have been proposed. Gene transferred stem cells that overexpress VEGF, if transplanted directly, can avoid the systemic injection route but may have less angiogenic potential since cells need to survive before they can secrete VEGF. An alternative approach is to utilize VEGF-transfected macrophages to achieve targeting purpose, based on the fact that the inflammatory response following MI will attract macrophages to migrate to that area. Yan et al.²² observed improved neovascularization and cardiac function by this method. However, this method cannot be combined with any anti-inflammatory therapy. Targeted delivery achieved through biocompatible drug carriers has also been studied. Our group has developed long circulating immunoliposomes^{9, 14, 23–25} for

delivering VEGF to MI tissue and antivasular drugs to irradiated tumors. Natural polymeric carriers, such as alginate, can entrap VEGF when forming gels in the presence of Ca^{2+} ions and release VEGF slowly^{26, 27}. These carriers show low levels of toxicity and immunogenicity in humans however lack a viable targeting capability²⁸. In a recent study, Yang et. al. placed VEGF embedded alginate beads intrapericardially to achieve targeting²⁹. Similar results were obtained by a gelatin hydrogel microsphere system incorporating VEGF³⁰.

In this study, we combined the targeted VEGF delivery using immunoliposomes and intramyocardial implantation of MSCs in MI tissue to treat MI. We chose intramyocardial injection of MSCs due to the fact that this route provides the highest cell survival and engraftment rate (~11%) as compared to other routes (intravenously ~3%, intracoronary ~3–6%)⁵. Previously we have shown that there is a strong inflammatory response during the first few days after MI providing a less than optimal microenvironment for cell survival, and that the optimal window for stem cell therapy may be within the second week of MI development³¹. Therefore, we took advantage of the inflammatory response to selectively deliver VEGF to the infarcted site using anti-P-selectin coated immunoliposomes. We choose anti P-selectin as our targeting moiety since our previous study showed that this adhesion molecule is significantly upregulated within 24 hours after MI⁹. Therefore, in this study, VEGF treatment was given immediately after ligation. Due to the fact that VEGF's plasma half-life is very short (only a few minutes)³², direct measurement of VEGF in the MI area may be extremely difficult, if not impossible. However, previously we have used radiolabeling to show that a significant number of anti-P-selectin conjugated immunoliposomes accumulate in the infarct region after MI¹⁴, and that VEGF encapsulated immunoliposomes targeting P-selectin significantly increase vascularity and oxygenation in the MI region⁹.

Our Picrosirius Red staining results indicated the least amount of collagen formation in the VEGF + MSC treated animals as compared to VEGF only, MSC only or untreated (MI only) group. We hypothesize that VEGF induced angiogenesis, as indicated by formation of anatomical blood vessels in Figure 4, may be responsible for the reduced collagen formation in the VEGF + MSC treated animals. Instead of measuring the number of the perfused vessels, which may vary with the change of the cardiac cycle, we used CD31 staining to measure total anatomical blood vessels, which does not change with the change of cardiac cycle. It has been shown that in transverse, but not longitudinal sections of the viable LV wall, capillary density can be an adequate measurement for the number of capillaries per tissue area^{33, 34}.

Various *in vitro* and *in vivo* studies have shown that stem cell therapy can partly restore heart function^{35, 36}. However, these improvements in cardiac function are for the most part small and transitory in nature due in part to the inefficient differentiation of the transplanted stem cells into cardiomyocytes³⁷. In this study, the VEGF + MSC combination treatment was shown to significantly improve the integration of stem cells into the host cardiac tissue after MI and myocardial repair through stimulation of angiogenesis. However, detailed assessments of survival of transplanted cells are required to better assess the percentage of transplanted cells that integrates into the MI tissue. Consistent with recent reports in the

literature^{38, 39}, our results indicate that for the most part differentiated MSCs exhibited characteristics of vascular cells but not cardiomyocytes, supporting the hypothesis that improvement of cardiac function after MSC therapy may be more related to incorporation of the transplanted stem cells into blood vessels than replacement of cardiomyocytes.

Conclusion

The strategy employed in this study integrates the advantages of active targeting using immunoliposomes and the highest stem cell survival rate from intramyocardial injection route, resulting in the most promising therapeutic strategy. We have shown that this strategy results in a significantly larger recovery in cardiac function compared to either VEGF only or MSC only treatment. This recovery was achieved primarily due to targeted VEGF-stimulated angiogenesis and not from replacement of cardiac myocytes by transplanted cells. Our targeted drug delivery system can also be used to deliver anti-inflammatory agents such as Cathepsin G⁴⁰ to further improve the local microenvironment and/or growth factors such as TGF- β ⁴¹ to assist cardiac myocyte differentiation.

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Figure 1.

Cardiac function loss over time represented by FS change. Y-axis is calculated by subtracting the FS at 4 weeks post-MI from the FS at 1 week post-MI. “*” significant difference compared to “Untreated”, by ANOVA, $p < 0.001$. “**” significant difference compared to “Untreated”, “VEGF” and “MSC”, by ANOVA, $p < 0.001$.

Figure 2. Collagen formation after MI. Untreated (A), targeted VEGF treated (B), MSC treated (C), and targeted VEGF + MSC treated (D). Heart sections were stained with Picrosirius Red. Images were taken using a circular polarizer. The scale bars represent 100 μm.

Figure 3. Percentage of collagen in untreated and treated MI heart sections based on Picrosirius Red staining. “*”, significant difference compared to “Untreated” group, by ANOVA, $p < 0.001$.

Figure 4.

Anatomical blood vessel regeneration after each treatment. Top panel: CD31 staining was used to detect anatomical vessels in sham operated (no MI) (A), untreated (B), VEGF treated (C), MSC treated (D), and VEGF + MSC treated rats (E) measured 4 weeks after MI induction. Blood vessels were stained, imaged and processed to count the number of blood vessels. The anatomical blood vessel density was then calculated by dividing the number of vessels by the area. The scale bars represent 100 μ m. Bottom panel: The anatomical blood vessel density, quantified from the top panel. The Y axis “Ratio” was calculated by normalizing the vessel density in the MI region of each heart sample to that in the non-MI region of the same sample. Data are presented as “mean + standard error” (n = 4 samples for each group). “*” significant difference compared to “Untreated” group, by ANOVA, $p < 0.01$.

Figure 5. MSCs in the MI region as indicated by GFP fluorescence three weeks after transplantation. Images taken from (A) MSC treated, or (B) VEGF + MSC combination treated MI rats. The scale bars represent 100 μm .

Figure 6. MSC differentiation after VEGF + MSC combination treatment. Images were taken from the border zone of the MI region. Samples were stained with different antibodies (A) anti-cardiac troponin T, or (B) anti- α -actinin, or (C) anti-vimentin, or (D) anti- α -smooth muscle actin. Transplanted GFP expressing MSCs are shown in green. Cy3 labeled antibodies are shown in red. The scale bars represent 100 μ m.

Table 1

Total number and density of MSCs quantified in the MI area by their fluorescence (n = 4 samples per group).

Treatment	MSC Only	VEGF + MSC	Percentage Increase
Number	6147.25 ± 881.77	9210.50 ± 518.25	49.83% *
Density	2179.49 ± 182.14	3543.35 ± 332.55	58.45% **

Data are presented as mean ± standard error.

*
 $p < 0.05$;

**
 $p < 0.01$.