【データ管理システムの整備】

重症拡張型心筋症への bridge-to-transplantation / recovery を目指した革新的な心筋治療法は，難治性疾患をもつ患者様に適用されることになる．このような患者様の数は少ないため，わずかな情報漏えいが患者様特定につながり得る．したがって，患者様の個人情報を保護するために，本研究に特化したデータ管理システムを導入・整備した．

(倫理面への配慮)

本研究で開発した統計的デザイン及び方法論は，上記の通り，現在，「仮想的」臨床試験におけるシミュレーションでその性能を検証している段階であり，患者様から直接に得られた現実データには適用しておらず，倫理面への配慮は現時点では必要ないと考える．ただし，現在蓄積されつつあるデータ管理においては，個人情報のとり扱いに関する各種法令・告示・通知・倫理指針を遵守する．例えば，当該臨床研究から得られた患者のデータは，連結不可能匿名化等の適切な手段を通じて，本研究に必要のない個人を識別できるデータは切り捨てられた状態で研究分担者の手に渡るようになっている．

C. 研究結果

表に示すように，Scenario1 では，当然のことながら，真のモデルであるモデルAに基づく従来の連続再評価法 a が，治療水準が選択される割合，その治療水準で治療される患者数が最も大きかった．ただし，モデル B に基づく従来の連続再評価法 b を適用した場合，治療水準が選択される割合，その治療水準で治療される患者数は著しく低くなった．すなわち，臨床医らが設定した，治療水準と有害事象発現確率との関係構造に関するモデルが誤規定された場合には，最適な治療水準で治療が実施されない可能性がある．Scenario2 に関しては，Scenario1 と逆の結果が得られた．

本研究で開発したデビアンス情報量規準 (DIC-based) に基づくモデル選択型連続再評価法は，モデル A に基づく連続再評価法 a ほど性能は優れていないが，モデルを誤規定した場合に相当するモデル B に基づく連続再評価法 b よりも相当に性能がよいことが示唆された．すなわち，モデル選択型連続再評価法を用いれば，モデルの誤規定があった場合でも，治療水準と有害事象発現確率との真の関係構造に近いモデルを選択し，その選択されたモデルに基づいて各患者様への最適な治療水準を割り付け，各治療水準に対する有害事象発現確率を推定し得る．

D. 考察

モデル選択型連続再評価法は，臨床医によりモデルが誤規定されたとしても，治療水準が選択される割合，その治療水準で治療される患者数において頑健な結果をもたらすことが示唆された．また，表に示すように，有害事象発現確率が高いほど，治療水準と有害事象発現確率との真の関係構造に近いモデルを選択することができそうである．

E. 結論

本研究で開発した統計的デザイン・方法論は，従来のデザイン・方法論と比較して頑健であり，実地での適用可能性の視点からみても活用できそうである．ただし，順序付けられた治療水準に対して有害事象発現確率が単調に増加することを前提としており，重症拡張型

心筋症への bridge-to-transplantation / recovery を目指した革新的な心筋治療法における安全性に関する臨床評価でその前提が崩れる場面も，治療水準と有害事象発現確率との真の関係構造が未知であるが故に皆無とはいえない．次年度以降は，上記の場面も含めて包括的なシミュレーションを実施し，新しい生物統計学デザイン・方法論の開発を実施し，重症拡張型心筋症への bridge-to-transplantation / recovery を目指した革新的な心筋治療法の臨床評価における基盤の確立に寄与したい．

F. 健康危機情報

該当せず

G. 研究発表

1. 学会発表

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5)

H. 知的財産権の出願・登録状況

(予定も含む)

1. 特許取得

該当せず.

2. 実用新案登録

該当せず.

3. その他

該当せず.

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分担研究報告書

cGMP対応の細胞培養システムの構築

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研究要旨

自己筋芽細胞や脂肪幹細胞を用いた再生医療の普及を図るためには、細胞培養施設(CPC)で製造される細胞製剤の薬事対応を担保するCPC図書群の作成が必須である。本研究では、CPC図書作成に必要な施設・製造・品質保証要件を検討する。

A. 研究目的

自己細胞を用いた再生医療の普及を図るためには、薬事対応のCPC図書の作成が必須である。本研究では、CPCで実際の細胞培養を行いCPC図書作成に必要な標準管理基準書・手順書の作成を行った。

B. 研究方法

先端医療センターが保有するCPCでヒト脂肪細胞・筋芽細胞を用いてCPC Cold runを実施し、そのデータを用いて細胞製造・品質保証に必要なCPC図書を作成した。

(倫理面への配慮)

非臨床研究である。動物実験は実施していない。使用したヒト細胞は、市販されおり、ICが取得され、個人情報の管理がされている試料を使用した。

C. 研究結果

細胞培養後の出荷判定基準設定と逸脱時の手順設定はCPC図書における最重要項目の一つである。本年度は、出荷判定基準自体のバリデーションと設定値の再設定が可能となるような文章体系を策定した。

D. 考察

CPCでCold Runを実施することで、実際の臨床に供する培養細胞の細胞規格と出荷判定基準の設定が可能となった。

E. 結論

本研究では、幹細胞研究指針適合確認に基づく臨床研究、高度医療実施、それに続く医師主導治験を実施するうえで必要とされるCPC 図書文書体系の提供を目指しており、わが国の再生医療研究の進展を促進するものと考えている。

G. 研究発表

1. 論文発表

なし

2. 学会発表

なし

H. 知的財産権の出願・登録状況

(予定を含む。)

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

Ⅲ. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shigeru Miyagawa	Impaired myocardium regeneration with skeletal cell sheets-A pre-clinical trial for tissue engineered regeneration therapy	Transplantation		In press	2010
Hanayuki Okura	Properties of Hepatocyte-Like Cell From Human Adipose Tissue-Derived Mesenchymal Stem Cells	Tissue Engineering Part C		In press	2010
Naosumi Sekiya	Layered implantation of myoblast sheets attenuates adverse cardiac remodeling of the infarcted heart	Journal of Thoracic Cardiovascular Surgery	Oct;138(4)	985-93	2009

Impaired myocardium regeneration with skeletal cell sheets-A pre-clinical trial for tissue engineered regeneration therapy-

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Key words; cells, heart failure, transplantation, tissue engineering

Footnotes

Shigeru Miyagawa participated in the writing of the paper. Atsuhiro Saito participated in research design. Taichi Sakaguchi and Yasushi Yoshikawa participated in data analysis. Takashi Yamauchi, Yukiko Imanishi, Naomasa Kawaguchi, and Noboru Teramoto participated in the performance of research. Nariaki Matsuura, Hidehiro Iida, Tatsuya Shimizu, Teruo Okano, and Yoshiki Sawa participated in research design.

Abbreviations

LAD left anterior descending

HE Hematoxylin eosin

SCs Skeletal cells

LV Left ventricle

ECM Extra cellular matrix

FAS Fractional area shortening

CK Color kinesis

PET Positron emission tomography

ESA End systolic area

PTF Perfusable blood flow

MBF Myocardial blood flow

BM-MNCs Bone marrow-mononuclear cells

HGF Hepatocyte growth factor

DCM Dilated cardiomyopathy

Abstract

[Background] We hypothesized that autologous skeletal cell (SC) sheets regenerate the infract myocardium in porcine heart as a pre-clinical trial.

[Methods and Results] The impaired heart was created by implantation of ameroid constrictor on LAD for 4 weeks. SCs isolated from leg muscle were cultured and detached from the temperature responsive domain coated dishes as single monolayer cell-sheet at 20° C. Following therapies were conducted: SC sheets (SC group, n=5); Sham (C group n=5). Echocardiography demonstrated that cardiac performance was significantly improved in the SC group 3 and 6 months after operation (Fractional area shortening, 3 months; SC vs C=49.5±2.8 vs 24.6±2.0%, $P<0.05$) and LV dilatation was well attenuated in the SC group. Color Kinesis Index showed that distressed regional diastolic and systolic function in infarcted anterior wall was significantly recovered (SC vs C=57.4±8.6 vs 30.2±4.7%, $P<0.05$, diastolic: 58.5±4.5 vs 35.4±6.6%, $P<0.05$, systolic). Factor VIII immunostains demonstrated that vascular density was significantly higher in the SC group than the C group. And % fibrosis and cell diameter was significantly lower in the SC group. And HE staining depicted that skeletal

origin cells and well developed-layered smooth muscle cells were detected in the implanted area. Positron emission tomography showed better myocardial perfusion and more viable myocardial tissue in the distressed myocardium receiving SC sheets compared with the myocardium receiving no sheets.

[Conclusions]

SC sheet implantation improved cardiac function by attenuating the cardiac remodeling in the porcine ischemic myocardium, suggesting a promising strategy for myocardial regeneration therapy in the impaired myocardium.

Key words: Cells, heart failure, myocardial infarction, tissue, transplantation

Introduction

Despite the recent remarkable progress in medical and surgical treatments for heart failure, end-stage heart failure has been still a major cause of death worldwide. After myocardial infarction, the myocardium is capable of a

very limited regenerative capacity and no medication or procedure used clinically has shown efficacy in regenerating myocardial scar tissue with functioning tissue. Thus, there is a need for new therapeutics to regenerate damaged myocardium.

Recent developments in tissue engineering show promise for the creation of functional cardiac tissues without the need for biodegradable alternatives for the extra cellular matrix (ECM) [1]. And we reported that cardiomyocyte sheets have been developed by using temperature responsive culture dishes and these sheets survived in the back of nude rats and showed a spontaneous contraction over a long period time [2]. Recent reports suggested that cardiomyocyte sheets integrated with the impaired myocardium and improved cardiac performance in a rat model of ischemic myocardium [3]. And more recently, in the aim of clinical application, non ligature implantation of skeletal myoblast sheet regenerated the damaged myocardium and improved global cardiac function by attenuating the cardiac remodeling in the rat ligation model [4] and dilated cardiomyopathy hamster model [5]. This cell delivery system by using cell sheets implantation showed better restoration of damaged myocardium compared

with needle injection [4, 5]. Moreover grafting of skeletal myoblast sheets attenuated cardiac remodeling and improved cardiac performance in pacing-induced canine heart failure model [6].

Given this body of evidence, we hypothesized that the autolougus skeletal cell sheet implantation might remodel the chronic heart failure caused by ischemic injury.

Therefore, this preclinical study using Swine model was designed to test therapeutic effectiveness.

Method

Myocardial Infarction Model

"Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resource and published by the National Institutes of Health (NIH Publication No.86-23, revised 1985). This animal experiment was approved by the Animal Care Committee of Osaka university graduate school of medicine. We induced

acute myocardial infarction of 10 swines (20kg, KEARI, Japan) by the following method. Swines were preanesthetized by intramuscular injection of ketamine hydrochloride 20 mg/kg (Ketalar, Sankyo) and xylazine 2 mg/kg (Seractar, Bayer). Animals were positioned spine, and a 22-gauge indwelling needle (Surflo F&F, Terumo, Tokyo, Japan) was inserted in the central vein of the auricle. A three-way cock (Terufusion TS-TR2K, Terumo, Tokyo, Japan) was attached to the external cylinder of the indwelling needle, and an extension tube was connected for continuous anesthetic injection. The animals were intubated with an endotracheal cannula (6Fr, Sheridan) using a pharyngoscope and then connected to an artificial respirator (Harvard, USA) by the cannula. Artificial respiration was implemented at a stroke volume of 200-300 mL/stroke and a stroke frequency of 20/min. The animals were continuously drip injected with propofol 6 mg/kg/hr (Diprivan, AstraZeneca) and vecuronium bromide 0.05 mg/kg/hr (Musculux, Sankyo Yell Yakuhin Co., Ltd., Japan) using a syringe pump (Terufusion TE-3310N, Terumo, Japan). The animal was then fixed in a recumbent position so that the left thorax was exposed, and the outer layer of skin and muscles between the third and fourth ribs were

dissected. After confirming the cutting into the thoracic cavity, the distance between the third and fourth ribs was widened with a rib spreader to allow a direct view of the left auricle and the left anterior descending coronary artery (LAD). The pericardium was dissected along the LAD from the upper part of the left auricle (about six cm.) to expose the myocardium around the LAD. LAD on the proximal side below the left auricle from the myocardium was exfoliated for approximately 1 cm, and then a small amount of lidocaine hydrochloride jelly (Xylocaine jelly, AstraZeneca) was applied to allow for anesthetizing the area. An ameroid constrictor (COR-2.50-SS, Research Instruments) was then fit using No.1 or 2 suture. The chest cavity was closed to end the procedures. The animals were randomly divided into two treatment groups: The first received autologous skeletal cell (SC) sheet implantation (SC group, n = 5). For control, we have done sham operation (C group, n = 5)

Preparation of skeletal cell sheets for Grafting

1 week after implantation of ameroid constrictor on LAD, skeletal muscle weighing about 5grams was removed from the pre-tibial region with the porcine under general anesthesia. Following the addition of trypsin-EDTA

(Gibco, Grand Island, NY), excessive connective tissue was carefully removed to minimize the content of contaminating fibroblasts, and the muscle tissue was minced until the fine pieces formed a homogeneous mass. The specimens were then incubated at 37°C in shaker bath with 0.5% type 1 colllagenase (Gibco) in Dulbecco's modified Eagle's medium (Gibco). After brief placement, the fluid was collected, and the same volume of culture medium, SkBM (Cambrex, Walkersville, MD) supplemented with fetal bovine serum (Thermo Trace, Melbourne, Australia), was added to halt the enzymatic digestion process. The cells were collected by centrifugation, and the putative skeletal cells were seeded into 150 cm^2 polystyrene flasks following removal of fibroblasts by sedimentation for a few hours and cultured in SkBM at 37°C. During the culture process, we maintained cell densities at <70% confluence by carrying out passaging of cells for one time to prevent skeletal cells from premature differentiation and fusion process resulting in myotubes formation. When the cells become approximately 70% confluent after 10 to 11days cultivation, the cells were dissociated from the flasks with trypsin-EDTA and re-incubated on 100mm tempreture-responsive culture dishes (Cellseed, Tokyo, Japan) at 37°C with

the cell numbers adjusted to 1×10^7 per dish. More than 90% of these cells were desmin positive (Figure 1). After 4 days, the dishes were removed to refrigerator set at 20°C, and left there for approximately 30 minutes. During that time the SC sheets detached spontaneously from the surfaces. Each sheet had a diameter of 30-40mm and consisted of layers of skeletal cells (SCs); the sheets were approximately 100μm thick in cross-sectional views (Figure 1). Approximately 10 sheets were obtained from the 5 grams of skeletal muscle.

Implantation of skeletal cell sheets

Autologous SC sheet implantation was performed in the swine 4 weeks after LAD ligation. Swin were anesthetized as mentioned above. The swines were exposed through the sternum. The infarct area was identified visually on the basis of surface scarring and abnormal wall motion. In the SC group, we implanted 10 SC sheets into the infarcted myocardium. The control group was treated similarly but received no SC sheets. Since pilling up four or more sheets caused the central necrosis of the myoblasts presumably because the lack of in oxygen supply, we decided to pile two or three layers of the SC sheet over the broad surface of the impaired heart.

Measurement of cardiac function

Swin were anesthetized as mentioned above. Cardiac ultrasonography was performed with a commercially available echocardiograph, SONOS 5500 (PHILIPS Medical Systems, USA). A 3–MHz annular array transducer was placed on a layer of acoustic coupling gel that was applied to the left hemithorax. Swines were examined in a shallow left lateral decubitus position. The heart was first imaged in the two-dimensional mode in short-axis views at the level of the largest left ventricle (LV) diameter. The calculation of the LV volume was based on the LV short axis area using AQ system [7]. And Fractional area shortening (FAS) of the LV Diastolic was calculated as follows:

FAS(%)=[(LV end diastolic area-LV end systolic area)/LV end diastolic area] ×100

These data are presented as the average of measurements of two or three selected beats.

Quantification of regional diastolic and systolic function by Color kinesis

Diastolic CK images were obtained using a commercially available ultrasound system (SONOS 5500, Philips Medical Systems) from the LV

midpapillary short-axis view for the determination of wall motion asynchrony as previously reported [8]. CK examined every image pixel within the region of interest, which was drawn around the LV cavity, classifying it as either blood or tissue based on integrated backscatter data. During diastole, each pixel was tracked into the next frame, and pixel transitions from endocardium to blood were detected and interpreted as diastolic endocardial motions. These pixel transitions were encoded using a color hue specific to each consecutive video frame, so that each color represents the excursion of that segment over a 33-ms period of time. The sites of regional LV diastolic wall motion or regions of interest were set on the basis of standard segmentation models: anterior, lateral, posterior, inferior, anteroseptal wall. The CK diastolic index was defined as the LV segmental filling fraction during the first 30% of the diastolic filling time (LV segmental cavity area expansion during the first 30% of diastole, divided by the segmental end-diastolic LV cavity area expansion, expressed as a percentage). We introduced the use of Color kinesis method that displays endocardial motion in real time to evaluate the regional systolic function [8].

Histopathology

LV myocardium specimens were obtained 6mo after the skeletal cell sheet implantation. Each specimen was fixed with 10% buffered formalin and embedded in paraffin. A few serial sections were prepared from each specimen and stained with hematoxylin eosin stain and Elastica Masson Goldner for histological examination or with Masson Trichrome stain to assess the collagen content.

To label vascular endothelial cells so that the blood vessels could be counted, immunohistochemical staining of factor VIII-related antigen was performed according to a modified protocol. Frozen sections were fixed with a 2% paraformaldehyde solution in PBS for 5 minutes at room temperature, immersed in methanol with 3% hydrogen peroxide for 15 minutes, then washed with PBS. The samples were covered with bovine serum albumin solution (DAKO LSAB Kit DAKO CORPORATION, Denmark) for 10 minutes to block nonspecific reactions. The specimens were incubated overnight with an EPOS-conjugated antibody against factor VIII-related antigen coupled with HRP (DAKO EPOS Anti-Human Von Wille brand Factor/ HRP, DAKO Denmark). After the samples were

washed with PBS, they were immersed in diaminobenzidine solution (0.3 mg/ml diaminobenzidine in PBS) to obtain positive staining. Ten different fields at 200× magnification were randomly selected, and the number of the stained vascular endothelial cells in each field was counted under a light microscope. The result was expressed as the number of blood vessels per square millimeter.

The following antibodies against smooth muscle cells and skeletal myosin (slow) were used to evaluate the existence of skeletal cells: Primary antibodies, anti-smooth muscle actin (clone 1A4, DAKO) anti-skeletal myosin(slow) (clone NOQ7.5.4D, Sigma); secondary antibodies, anti-mouse Ig bioptinylate (DAKO).

Picoro-sirius red staining for the assessment of myocardial fibrosis or periodic acid-Schiff (PAS) staining for that of cardimyocyte hypertrophy was done as described [9].

Positron emission tomography procedure

We performed Positron emission tomography (PET) studies on pigs which were transplanted skeletal cell sheets and control by using ^{15}0-water and ^{18}F-FDG. The pigs were anesthetized by introduction of pentobarbital

followed by continuous inhalation of propofol (4 mg/kg/h) and were placed supine on the bed of the scanner. PET was performed using a Headtome-III tomograph (Shimadzu, Kyoto, Japan) and data was analyzed as described elsewhere [10].

Holter Electro cardiography

To evaluate arrhythmia we used holter ECG for 24hours. We checked arrhythmia by checking the number of ventricular premature beat after skeletal cell sheet implantation in myocardial infarction porcine (n=3).

Data analysis

Data are expressed as means ± SEM and subjected to multiple analysis of variance (ANOVA) using the StatView 5.0 program (Abacus Concepts, Berkeley, CA). Echocardiographic data were first analyzed by two-way repeated measurement ANOVA for differences across the whole time course, and one-way ANOVA with the Tukey-Kramer post-hoc test were used to verify the significant for the specific comparison at each time point. To assess the significance of the differences between individual groups concerning other numeral data, statistical evaluation was performed with an