NEW CULTURE MEDIUM CREATES IMMUNE TOLERANCE BETWEEN TWO ALLOGENEIC TISSUE CELLS.

Victor Alexander, PhD.; Anthony Passerini, PhD; Emir Hodzic, PhD., Biomedical Engineering Dept. and Dept. of Medicine &Epidemiology of Vet. Med. of UC Davis

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Abstract.

Our hypothesis creation immune tolerance between two allogeneic tissue cells based on the 2 facts of embryonic and fetal development:

1. Embryonic tissue differentiations and fetus are developing under physiologically high doses of Progesterone (Pgn.) which are exceeding 100-500 times in adult serum level of it. And pregnant female/fetus are immune tolerant to each others.

2. Liver is functioning as a hematopoietic organ and supporting differentiation of Hematopoietic Stem Cells in early fetogenesis.

After creation a principally new culture medium containing high dose of Pgn. and specific cytokines (Invention have been registered by UC Davis, November, 2008) we have discovered dual actions of Pgn. to primary Liver tissue cells: stimulatory effect to multiplication of Hepatocytes (Heps, albumin +) and suppressor effect to all non-parenchymal cells (albumin -) of the Liver tissue. In this condition Hepatocytes continued high speed multiplication in whole 3 months long period of experiments.

When we mixed 1 month old 2 allogeneic primary Liver tissue cells together in this new culture medium, Heps of both hepatic tissue cells mixed with each others and began to multiply in whole period of 2 months long experiment! But in control medium both tissue cells didn't grow and died almost completely in 2 months (fig.1 and 2).

Even more, when after 2 months we replaced Pgn. containing medium to Control culture medium in 1 month period, no detectable cell death have been observed, just multiplication rate of "small" Heps slowed down, but size of "small" Heps increased reaching to normal size Heps. We hypothesized that this very useful phenomenon is connected not just with passive suppression of immune cells of the Liver tissue by Pgn., but active cell fusion of Heps, which may be to create a new type of MHC-1 antigen which is acceptable to both allogeneic immune cells of Liver tissues.

This very effective in vitro model will be very useful to create immune tolerance between Embryonic Stem Cells (ESC) and recipients' immune cells, which could solve immune rejection problem of ESC.

Introduction

- 1. Embryonic tissue differentiations and fetus are developing under physiologically high doses of Progesterone which are exceeding 100-500 times in adult serum level of it. And pregnant female and fetus are immune tolerant to each others.
- 2. Liver is functioning as a hematopoietic organ and supporting differentiation of Hematopoietic Stem Cells in early fetogenesis.
- 3. There are 2 main types of Progesterone Receptors: PR-A and PR-B. They are the same gene products, but m-RNA of this gene in nucleus is splicing differently in the different conditions and in different cells. PR-A has been shown to act as a trans-dominant repressor of PR-B controlled transcription in a cell and promoter specific manner.
- 5. MHC-1 and MHC-2 are the main antigens which play a crucial roles in immune rejection of transplanted tissue.
- 6. Self reactivity is prevented by processes which occur during development, rather than being genetically preprogrammed.
- 7. Demi-Lee Brennan is an Australian citizen whose body, after a liver transplant, changed blood type and adopted the immune system of her donor. The result of this is that her body no longer attempts to reject the transplanted liver and she therefore does not need immunosuppressant medication.

Hypotheses

Our discovery: Dual effects of high dose of Progesterone on adult Liver tissue cells: stimulatory to long term multiplication of Hepatocytes and suppressor effects to all non-parenchymal cells maybe used to create effective Immune Tolerance between two allogeneic tissue cells in vitro and in vivo.

Under high doses of Progesterone PR-B may increase synthesis of Cyclins (B, A, E, D) in the Hepatocytes which can speed up cell division and create "small" Hepatocytes through shortening G-1 phase (see: explanatory slide 1).

High degree synthesis of Cyclins also can create meiotic Heps through shortening S phase of Cell Cycle and that way to create a condition of easy cell fusions in Heps with each-others and with two allogeneic Heps. It can create a new types of MHC-1 genes which are more acceptable to both immune cells of the two allogeneic liver tissues and that way create permanent immune tolerance between two allogeneic tissue cells.

Design of experiment and Methods.

- A new culture medium (NCM+P) containing Growth Factors(FGF-1, EGF, VEGF), high physiological dose of Progesterone and low dose of Lipopolysaccharide (LPS) has been designed .
- Three groups of in vitro culturing experiments were conducted. Every group include fresh harvested whole Liver tissue cells from 2 one month old normal mice (different strains) cultured in 4 culture flasks and 2 six well plates without separation to individual tissue cells(2 flasks + 1 six well plate with P+NCM and 2 flasks+ 1 six well plate with NCM-P). Both culture mediums in the flasks and six well plates were changed in every 3 days. Whole Primary Liver tissue cells seeded in the flasks and six well plates with P+NCM began to grow after 3-4 days of lag phase, which correlated with the concentration of Progesterone in the medium. In control medium (NCM-P) cells began to grow in the second day of culturing.
- Immunofluorescent anti-mouse albumin antibody staining have been used for checking of functional maturity & differentiation of Hepa from non-parenchymal liver tissue cells.
- Immunofluorescent anti mouse Flk-1; HAS-2; TERT; VE-Cadherin; β-Catenin; CD-133 also have been used (results were not included for future Grant investigation and patenting).
- Glycerol+ Hoechst 33342 dye is used for nuclear DNA staining.
- Total RNAs have been isolated from experimental cultured cells and after synthesis of c-DNAs from m-RNAs, all genetic materials have been saved in freezer for following QRT-PCR analysis of Genes of interest (Alb; AFP; GAPDH; β-catenin; HAS-3; TERT; VE-Cad; Flk-1; Histon p-1; LAT; DMT). (results were not included).

Research Results

High dose Progesterone containing culture medium selectively stimulated growth & multiplication of Heps (albumin +; red fluorescence) and suppressed growth all non-parenchymal cells of Liver tissue in all 3 months long experiments. But all Heps disappeared in control culture medium in 1 month and only non-parenchymal Liver tissue cels (albumin-; no fluorescence) were seen in control plate after 3 months. (fig.1 and 2.)

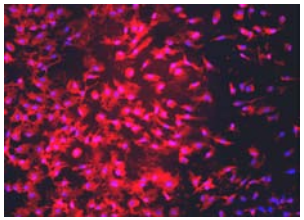


fig. 1

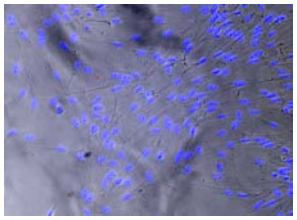


fig. 2

After discovery dual actions of high physiological dose of Progesterone on Heps and nonparenchymal (NP) cells of adult Liver tissue in vitro condition, we designed another experiment to check our hypothesis if high dose of Progesterone can create immune tolerance between two allogeneic Liver tissue cells.

We mixed one months old two allogeneic primary Liver tissue cells together in 6 well plates and added P+NCM culture medium (containing high dose of Progesterone) on the cells in 3 wells and another 3 wells we added the same medium, but without Progesterone as a control. After two months we fixed cells growing in the plates and stained them with fluorescent anti-albumin staining.

Albumin+ Heps in P+NCM grew and multiplied well in all 3 wells of culture plates in whole 2 months period, but all cells in NCM-P have died in this period (fig. 3 and 4).

After 2 months of growth of mixed allogeneic Heps & NPcells in P+NCM we changed culture medium to NCM-P for one month, no visible cell death was observed in one month period, just size of small Heps increased to normal size and suppressed NP cells began to multiply very slowly.

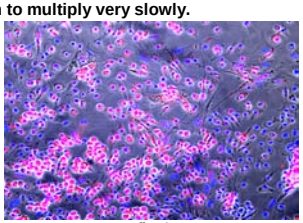


fig. 3

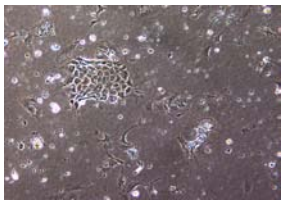


fig. 4

Discussion

Results of our first experiment have brought to discovery: Dual effects of high dose of Progesterone on adult Liver tissue cells: Selective stimulatory effect to Heps and suppressor effect to all non-parenchymal (NP) liver tissue cells. On the base of this discovery we have invented for the first time a very successful new Selective Culture Medium for long term culturing of primary Hepatocytes in which Heps have grown and multiplied in whole period of 3 months long experiment (Invention has been registered by UC Davis, November, 2008).

Our hypothetical explanation of Selectivity of Progesterone to different Liver tissue cells depends on different nuclear splicing of m-RNA of the same Progesterone Receptor (PR) gene in these cells, which bring to synthesis PR-B in Heps (activator of gene expressions) and PR-A (supressor of gene expressions) in NP cells (see: fig 5).

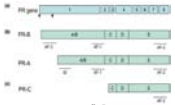


fig.5

Discussion (continuation).

Results of our second experiment have shown high dose of Progesterone create permanent in vitro immune tolerance between 2 allogeneic liver tissue cells.

It is very useful phenomenon and could bring to solution of immune rejection problem between Embryonic Stem Cells (ESC) and recipients' immune cells and eliminate last barrier using ESC in the clinics for curing many incurable diseases.

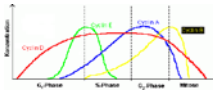
Mechanism of this immune tolerance phenomenon have to be investigated immediately and very seriously taking into account of crucial importance of this phenomenon for theoretical and practical Medicine!

Our hypothetical explanation of this phenomenon is next:

Heps which were activated by high dose of Progesterone and other components of P+NCM culture medium synthesize many Cyclins, which in turn are shortening G1 and even S phases of cell cycle. Under this condition Heps synthesize only minimal amount of necessary proteins and quickly undergo to cell division and it can create "small Heps", which we have observed (see: fig. 1 & 3).

High level of Cyclins even could decrease S phase of cell cycle which could bring to meiotic, 1n chromosomes containing Heps which could easily undergo to fusion with each-others. Under this condition if we mix two allogeneic liver tissue cells together (which we did) they could fuse with each-others randomly. Because of MHC-1 genes are predominant genes, these fusions will create new cells with mixed and different MHC-1 antigens. Some of these new fused Heps with different MHC-1 antigens will be acceptable to both non-parenchymal cells with immune properties of allogeneic Liver (see: explanatory fig. 1).

- Creation new Heps with more acceptable MHC-1 antigens and long term suppression of immune cells under high dose of Progesterone could bring to permanent in vitro (and it is possible in vivo also) immune tolerance between two allogeneic tissue cells, which we have observed!
- It is possible there are many unknown yet cellular mechanisms which are taking a part in this immune tolerance phenomenon. Only future fundamental investigations and collaborations could find out all mechanisms of unknown yet molecular process in and between cells which able to change our primitive yet understanding about interactions of cells and immunity!



Ex. Fig. 1

Conclusions:

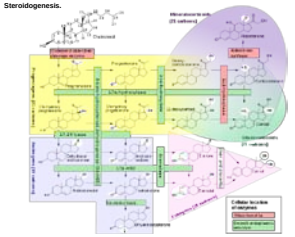
- 1. Dual effects of high dose Progesterone on the Heps and Non-parenchymal cells of the Liver tissue make it possible to create in the first time new types of selective culture mediums for long term culturing of Heps.
- 2. High dose of Progesterone creates in vitro permanent immune tolerance between two allogeneic liver tissue cells. Expanding of this experiment to different types of tissue cells and Embryonic Stem Cells (ESC) could solve immune rejection problem of ESC and revolutionize organ, cell and ESC transplantations.

Future Directions:

1. Invent a special culture medium for creation permanent immune tolerance between Embryonic Stem Cells (ESC) and recipients' immune cells using our novel know-how methodic approach. We will use a new culture medium together with special cellular system which will help to create not just only immune tolerance, but also will promote differentiation of ESC to desired tissue cells.
2. Prove an existence of a special reverse pathway in some immune cells which gives quick information from protein to synthesize special gene segment in DNA through synthesis of complementary RNA and this mechanism could be fast defense mechanism of mammals to environmental hazards (infection and cancer).

Explanatory slide 2

Steroidogenesis.



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Contact address:

Dr. Alexander Victor J., MD., PhD.,
The President of Capital Stem Cell Research Co.
2819 Toronja Way, Sacramento, CA 95833
www.capital-research-corporation.com
E-mail: vja@surewest.net
Phone/Fax: 916-920-1730