Induction of Differentiation of Adipofibroblasts Using a Defined Treatment Medium without DMI

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Abstract

The induction of differentiation of fat cell precursors in vitro has traditionally been accomplished by exposure of the cells at confluence to a mixture of dexamethasone, 1-methyl-3-isobutylxanthine, and insulin (DMI). In our laboratory, we treated ovine adipofibroblasts with three different defined media to determine which one would optimize the induction of differentiation without using DMI. A defined medium containing insulin, transferrin, triiodothyronine and hydrocortisone (ITTC) promoted the most differentiation in the cultured cells. Subsequently, we utilized ITTC to investigate the effects of plating density, substratum and the timing of treatment application on subsequent lipid conversion of ovine adipofibroblasts. Two protocols resulted in similar levels of differentiation: (1) plating at a density of 20,000 cells per well and allowing the cells to proliferate to confluence in a serum-containing medium before the application of ITTC or (2) plating at a density of 100,000 cells per well and adding the ITTC treatment immediately after a 24 hour attachment period in a serum-containing medium. Coating the wells with a substratum of pig skin gelatin before plating resulted in a small increase in the amount of differentiation over that seen in uncoated wells. A preliminary study testing the effect of a thiazolidinedione (T-174) on subsequent lipid production in adipofibroblasts determined that this chemical enhances differentiation. These studies suggest that an optimal defined treatment medium can be formulated without DMI to induce the differentiation of adipofibroblasts to adipocytes.

Key words: adipocytes, adipofibroblasts, defined media, differentiation, preadipocytes.

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Methodology for the *in vitro* culture of adipose tissue emerged in the late 1950's when Trowell incubated small pieces of fat from rats in a chamber apparatus that maintained the tissue in a synthetic medium and an atmosphere of 5% CO_2: 95% O_2 [30] (Fig. 1). Subsequent variations on this explant culture technique were utilized by other laboratories with the primary purpose of simply maintaining the adipose tissue for experimental studies on regulation and metabolism [11, 33]. Researchers discovered that when serum was added to the culture medium bathing the explants, fibroblast-like cells would grow out of the main fat structure and would ultimately accumulate cytoplasmic lipid [24].

At the same time that explant cultures were being used in adipose research, work was also initiated to isolate fat cells from the multicellular milieu of adipose tissue (Fig. 1). Scientists first used digestion with collagenase followed by centrifugation to separate free-floating fat cells from sedimented stromal-vascular cells [7, 21]. The suspended adipocytes were then available in a more purified population for metabolic studies. Gradually two divergent lines of fat cell research evolved focusing either on the characteristics of the stromal-vascular (SV) fraction or the adipocyte fraction.

Investigators who worked with the SV cells often referred to these cells as preadipocytes [3, 20, 22, 32]. Early in culture, SV cells resemble fibroblasts morphologically but ultimately assume the rounded appearance of mature adipocytes and accumulate intracellular lipid (differentiate) [6, 15, 17]. Elevations in levels of lipogenic enzymes such as lipoprotein lipase (LPL, early marker) or glycerol-3-phosphate dehydrogenase (GPDH, late marker) are characteristic of mature adipocytes and document that differentiation is occurring in the preadipocyte population [38, 39].

Researchers culturing the adipocyte fraction first had to solve the mechanical problem of anchoring the highly buoyant, free-floating fat cells to a fixed surface. They

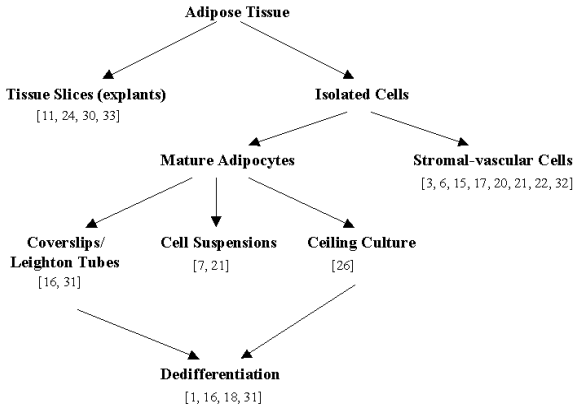

Figure 1. Flow diagram depicting the evolution of adipose cell research. The references listed are those cited in this paper and are only representative of the large number of papers published in this field of research.

used a variety of methods such as reducing the volume of culture media [1], sandwiching the cells between two coverslips [16, 31] or devising a technique called ceiling culture [26]. During culture *in vitro*, adipocytes were observed to lose their characteristic, lipid-filled morphology and assume a more fibroblast-like appearance, a process often termed dedifferentiation [1, 16, 18, 26, 31]. These dedifferentiated adipocytes were sometimes referred to as adipofibroblasts [1, 35].

Regardless of which type of preadipose cell was isolated, a common laboratory technique used to induce a differentiative response in these cells was to expose the cultures to an induction treatment called DMI (dexamethasone, 1-methyl-3-isobutylxanthine and insulin) [23]. In our laboratory we initially used DMI in a serum-based system to induce the differentiation of ovine adipofibroblasts [35]. However, this traditional delivery of DMI to the cells in a serum-containing medium added to the complexity of deciphering what regulatory agents were controlling the differentiative conversion process.

In efforts to minimize the complicating effects of serum in a medium, researchers developed defined media formulations, which allowed the determination of the effects of specific factors on cell cultures. However, it has been difficult to find a commonality in those factors found to induce *in vitro* differentiation in cultures of preadipose cells from different animal species. Working with porcine preadipocytes, Hausman [14] determined that insulin, insulin-like growth factor-I (IFG-I), and triiodothyronine (T_3) increased the differentiation of pig preadipocytes. Suryawan and Hu [27] demonstrated that insulin and hydrocortisone, but not T_3, were important in the differentiation of stromal-vascular cells in pigs. In the bovine system, the addition of a thiazolidinedione (T-174) to a defined medium containing transferrin, biotin, pantothenate, insulin and a lipid mixture resulted in an increase in the number of lipid-filled adipocytes [19]. Researchers initially attempting to induce differentiation in cultures of sheep fibroblasts developed a defined medium composed of insulin, dexamethasone, a lipid preparation and fibroblast growth factor (FGF) [4]. In our laboratory, we initiated research to develop a defined medium that would induce differentiation in sheep adipofibroblasts without DMI [36]. More recently, Soret and al. [25] ascertained that a combination of insulin, triiodothyronine, dexamethasone, and rosiglitazone (a thiazolidinedione) was effective in promoting differentiation in ovine preadipocytes. This paper provides additional information about the effects of various induction agents and protocols on the differentiation of ovine adipofibroblasts.

Materials and Methods

Materials

All cell culture plasticware were supplied by Nunc (Naperville, IL, USA). Dulbecco's modified Eagle medium (DMEM), Ham's F-12 medium, fetal bovine serum (FBS), penicillin-streptomycin, gentamicin, insulin, and trypsin were purchased from GibcoBRL (Rockville, MD, USA). Triiodothyronine, transferrin, pig skin gelatin, hydrocortisone, hepes, pantothenate, dexamethasone, 1-methyl-3-isobuylxanthine, Giemsa, oil red O and biotin were obtained from Sigma (St. Louis, MO, USA). Basic

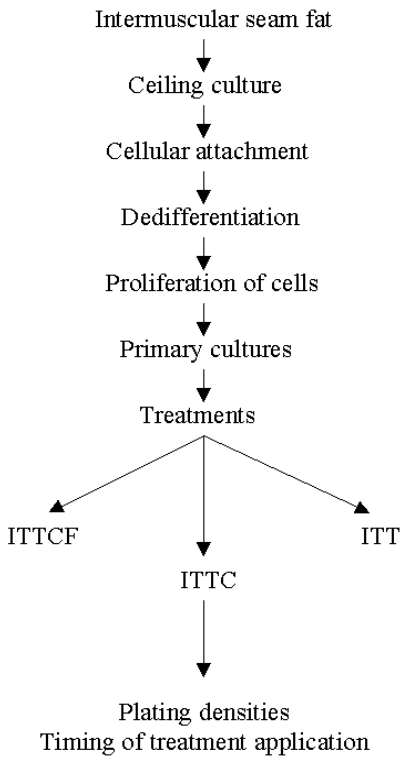

Figure 2. Flow diagram delineating the protocols for the isolation of adipofibroblasts and subsequent experimentation.

fibroblast growth factor (bFGF) was purchased from Collaborative Biomedical Products/Becton-Dickenson (Bedford, MA, USA). T-174 was produced by Acros Organics/Fisher Scientific (Pittsburgh, PA, USA).

Animals and Cells

The ovine adipofibroblasts used in these studies were obtained from the intermuscular fat depot (located between the semimembranosus and semitendinosus muscles) of three sheep, each weighing approximately 130 pounds (five-six months of age). Cells were isolated by placing the finely minced fat tissue into 25 cm^2 cell culture flasks, completely filling the flasks with DMEM + 10% FBS, inverting the flasks (ceiling culture) and allowing the fat cells to attach to the top surface area [26, 35, 36]. After the attachment of the cells, the flasks were re-inverted to their normal positions, and the cells were propagated for future experimentation or cryopreservation (Fig. 2).

Cells of passage numbers one through four were used in these experiments and were plated into 24-well plates. Cultures were maintained at 37°C in a 5% CO_2 incubator. Before being placed on a defined treatment, all wells were first rinsed three times with DMEM. Control wells were maintained in DMEM + 10% FBS. Media were routinely changed every 48 hours. Terminal wells were fixed with 10% formalin and stained with oil red O, followed by a Giemsa counterstain [36]. Cells were evaluated microscopically for the presence of lipid, which appeared as red, intracellular, refractile structures.

Media

The underlying goal of our laboratory is to develop a defined medium formulation that will maintain both ovine adipofibroblasts and ovine satellite cells in a co-culture environment [9]. Three defined media (Table 1) were selected as possible candidates for the co-culture system. Exposure of ovine satellite cell clones to ITT, a treatment containing insulin, transferrin and triiodothyronine, enhanced their subsequent differentiation/fusion [34]. However, ITT was not optimal for the culture of 3T3-L1 cells, a preadipocyte cell line which was initially used in our laboratory as a model for our fat cell system [9]. ITTCF, which includes insulin, transferrin, triiodothyronine, hydrocortisone and FGF [36] was successful in promoting the proliferation and fusion of both equine and wapiti satellite cells [5, 10]. In preliminary studies, ITTCF also appeared to be able to induce the differentiation of ovine adipofibroblasts [36]. The third defined medium selected was ITTC, which contains insulin, transferrin, triiodothyronine, and hydrocortisone (but no FGF) and was used successfully by Suryawan and Hu in culturing porcine preadipocytes [27, 28]. Our ovine adipofibroblasts had not been previously tested with ITTC.

Table 1. Formulations of three defined media.

Components	ITTCF	ITTC	ITT
Hepes			15 mM
Panthothenate			17 µM
Biotin			33 µM
Insulin	850 nM	850 nM	0.5 µM or 0.5 x 10^{-6} M
T_3	0.2 nM	0.2 nM	0.2 nM (135 pg/ml)
Transferrin	10 µg/ml	10 µg/ml	10 µg/ml
Penicillin/Streptomycin	10 ml/L	10 ml/L	10 ml/L
Gentamicin	5 ml/L	5 ml/L	5 ml/L
FGF	25-100 ng/ml*		
Hydrocortisone	50 ng/ml	50 ng/ml	

*The original formulation called for 100 ng/ml of FGF.

Table 2. Treatment of ovine adipofibroblasts with three defined media.

Defined Medium	Cell health and morphology	Differentiation
ITT	Small amount of cell detachment, moderate # of cells with vacuoles	none
ITTCF	Dense, intact monolayer with some areas of piling up; moderate # of cells with vacuoles	+
ITTC	Small amount of cell detachment, cells appear healthy	++

\+ minimal amount of differentiation
++ small amount of differentiation

Experimental design

Adipofibroblasts were treated with the three different defined media to determine which was the most effective at inducing differentiation. Ovine cells were plated at a density of 20,000 cells per well, allowed to proliferate to confluence (approximately five days), and then exposed to the treatments for periods ranging from eight to fourteen days. The wells were then fixed and stained and observed for morphological evidence of differentiation (accumulation of lipid as evidenced by oil red O staining).

After the optimal defined medium for differentiation induction was determined, experiments were conducted to examine the effect of plating density, substratum and timing of the application of treatment on subsequent differentiation. Cultures of cells from three sheep were plated in 24-well plates at either 20,000 cells per well (100 cells/mm^2) or 100,000 cells per well (500 cells/mm^2), a density that simulates confluence. Two different timing strategies for treatment application were used. For those cultures plated at an initial density of 20,000 cells per well, treatment was applied when the cells reached a confluent state, which required approximately five days of growth in a serum-containing medium. In wells plated with 100,000 cells per well, cultures were placed on treatment immediately after the 24 hour attachment period. Cells at both plating densities were also exposed to either a substratum of pig skin gelatin (PSG; 0.01%) or plastic. PSG was selected as the test substratum because it had been previously determined to be the optimal substratum for the culture of ovine satellite cells [8]. After application, cultures were exposed to the defined treatment for a period of seven to thirteen days before fixing and staining.

Results and Discussion

Achieving a state of growth arrest, by reaching confluence for example, appears to be necessary in order for a preadipocyte to withdraw from its cell cycle and begin to produce lipid [2, 12]. Previous studies in our laboratory indicated that the defined media we tested were unable to support the proliferation of ovine adipofibroblasts from a density of 20,000 cells/well to a con-

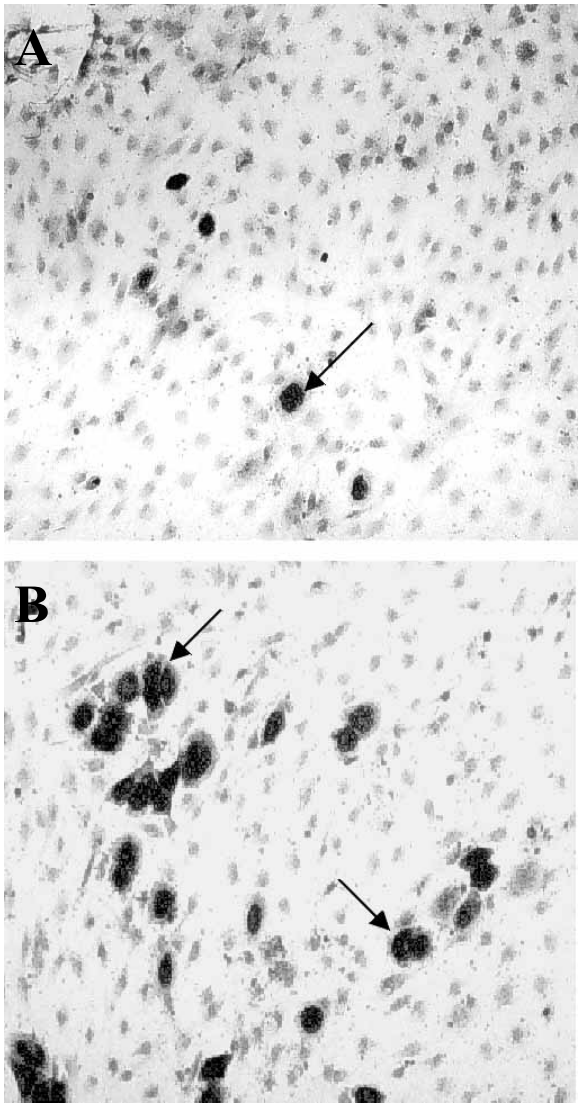

Figure 3. Adipofibroblasts plated at a density of 20,000 cells per well on (A) uncoated plasticware and (B) pig skin gelatin. Photomicrographs (100X) were taken at nine days post treatment application and indicate a small increase in differentiation on the PSG substratum. The differentiated cells (arrow denotes a few representative cells) appear darker due to the staining of lipid by oil red O.

Table 3. Treatment of ovine adipofibroblasts with ITTC defined medium.

Density: cells/well	Treatment Added At:	Health of cells		Differentiation[a]	
		Plastic[b]	PSG[c]	Plastic[b]	PSG[c]
20,000	Five days	d	d	1.3	2.8
100,000	24 hours	e	e	1.3	3.0

a In %
b Cells plated directly on plastic of cell culture plates
c Cells plated on a substratum of pig skin gelatin (0.1%).
d Small amount of cell detachment, cells appear healthy
e Small amount of cell detachment, a few areas with dense clumps of cells, cells overall appear healthy

fluent state of approximately 100,000 cells per well (unpublished observations). Therefore, cells plated at the lower density were allowed to proliferate to confluence in DMEM + 10% FBS for five days before being placed on treatment.

Of the three defined media tested with ovine adipofibroblasts, none gave optimal results in all of the categories of cell health, morphology and differentiation (Table 2). With the ITT treatment, the cells acquired vacuoles and did not display a differentiative morphology. After being exposed to ITTCF, the cells formed a dense monolayer which contained a small number of areas where the cells formed dense clumps [37], perhaps due to the effect of the proliferative growth factor, FGF. A minimal amount of differentiation was observed with ITTCF. The ITTC treatment induced a small amount of differentiation and appeared to be the best choice of the three media tested. Although a small amount of cell detachment occurred, the majority of the cells appeared to be healthy and were able to differentiate to a small degree.

The effect of plating density and substratum on subsequent differentiation of ovine adipofibroblasts when exposed to ITTC is summarized in Table 3. Plating cells on plastic at an initial density of 20,000 cells per well, with treatment application occurring at day five, resulted in a small amount of differentiation (Table 3). A similar amount of differentiation occurred when 100,000 cells were plated per well (on plastic), and the ITTC treatment was applied immediately after a 24 hour attachment period. Experiments investigating the effects of a substratum on the health and differentiation of adipofibroblasts indicated an increase in the final percentage of differentiation when PSG was used (Table 3, Fig. 3). Future studies will incorporate a pig skin gelatin substratum and seek to optimize the formulation of ITTC to improve cell attachment and increase differentiation.

The addition of a thiazolidinedione has been effective in enhancing the differentiation of preadipocytes in a serum-containing system [29] and in a defined treatment medium [25]. Preliminary data from experiments testing the effect of adding a thiazolidinedione, T-174, to the ITTC treatment indicated an increase in morphological differentiation of the adipofibroblasts to ~8% (data not shown). Thiazolidiones have also been shown to affect myoblasts and cause them to convert to an adipogenic pathway [13]. Future experiments may investigate the effect of incorporating a thiazolidione such as T-174 into a defined treatment for the co-culture of myogenic satellite cells with adipofibroblasts.

Adipose accretion and intramuscular marbling relate directly to the carcass quality and marketability of a final meat product. Determining the mechanisms that regulate the ratio of fat to muscle in an animal has high priority for producers and, ultimately, for animal science researchers. However, present studies on isolated fat cells *in vitro* often utilize a semi-toxic and artificial agent (DMI) to induce the differentiation of confluent cultures. A better system for evaluating fat cell physiology would be to develop culture conditions that induce differentiation using factors found *in vivo* and also to utilize non-confluent cultures. The research presented here represents initial efforts on our part to develop such a system. Future research will focus on the quantitative evaluation of the effects of other endocrine, paracrine, and autocrine extrinsic factors on fat cell induction and differentiation *in vitro*.

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