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Flow Cytometric Analysis of Porcine Preadipocytes

J.T. Wright

Russell Research Center, USDA, ARS, Athens, Georgia 30613

Abstract In this report, conditions have been established for utilizing monoclonal antibodies and fluorescence activated flow cytometry in studying antigen expression by primary porcine stromal-vascular cells cultured under various conditions. Single cells were isolated from cultures maintained in DME/F12 medium containing 10% fetal bovine serum, 2% pig serum, and containing 2% pig serum and 10 nM dexamethasone supplemented with growth hormone (GH), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β). Flow cytometric analyses revealed that the proportion of cells expressing detectable levels of the AD-1 cell surface antigen was greater in cultures supplemented with 2% pig serum and 10 nM dexamethasone than in other media. In cultures, GH, TNF-α and TGF-β each inhibited lipid deposition, whereas TNF-α and TGF-β, but not GH, inhibited AD-1 antigen expression. Inhibition of lipid deposition as well as antigen expression by TNF-α and TGF-β was reversible, but inhibition of cluster formation by GH was not reversed upon removal from cultures. In summary, differential effects of factors on surface antigen expression by preadipocytes are detectable by flow cytometry. Flow cytometric analysis using monoclonal antibodies produced against key developmentally regulated cell surface antigens is potentially a powerful analytical approach to the study of adipocyte development.

Key words: FACS, pig, monoclonal antibody, immunofluoresence, adipocyte differentiation

Subcutaneous stromal-vascular (S-V) tissue is comprised of several cell types including adipocyte precursors in various stages of differentiation. Overt differentiation of preadipocytes (lipid deposition) is monitored easily by measuring lipogenic enzyme activities as well as by using various histological and histochemical techniques [1–5]. However, preadipocytes are not readily distinguishable from other cell types in tissues or in S-V cultures prior to overt cytodifferentiation as suitable markers that identify cells of the adipogenic cell lineage are lacking. Clonal lines have been extremely useful in determining some key regulatory steps comprising overt differentiation of preadipocytes [6–10]. However, it is not certain if any of the clonal preadipose cell lines exhibit properties that are related to commitment of cells toward the adipogenic lineage.

We have characterized the tissue distribution and ontogeny of cell surface [11,12] and cytoplasmic [1] antigens recognized by anti-porcine adipocyte monoclonal antibodies (MAbs). One MAb in particular (AD-1) detects a cell surface antigen on all adipocytes [11] as well as on a small proportion of cells from presumptive adipose tissues at 50 days of gestation in the pig prior to appearance of detectable lipid in tissue [12]. In S-V cell cultures, the AD-1 antigen is detectable on a subpopulation of cells prior to the appearance of lipid and, in older cultures, detectable expression becomes restricted to adipocytes [11]. Reactivity within adipose tissues is also detectable on capillary endothelial cells associated with fat cell clusters, suggesting a possible functional or even lineage relationship between adipocytes and endothelial cells. Although the function of the AD-1 antigen is unknown, the AD-1 antigen may be a candidate marker for studying the adipocyte lineage prior to expression of other more well characterized markers such as enzymatic markers associated directly with lipid deposition.

One approach commonly used for studying cell lineages involves analysis of expression of differentially regulated cell surface antigens using fluorescence-activiated flow cytometry. An expanding list of characteristics of cells, including cell surface antigen expression, can now be

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Address reprint requests to J.T. Wright, Russell Research Center, USDA, ARS, Athens, GA 30613.

measured rapidly and accurately using flow cytometry (for review see [13]). In general, flow cytometric measurements are based on the type of interference detected as cells pass singly through the path of a laser. As cells pass through the laser, light diffracted by the cell or forward light scatter is related to cell size, and light reflected and refracted at a right angle to the forward path of the laser or 90° light scatter is an indication of the granularity or complexity of the cell. Multiple parameters are obtained for each cell analyzed, and approximately 20,000 events, or passages of particles through the laser, are typically analyzed for any given sample. The fraction of cells bearing a given antigenic marker in a sample properly labeled with monoclonal antibodies and fluorescent second antibodies is determined by a fluorescence distribution histogram. Fluorescence is sometimes coupled to cell size and granularity distribution data as well as other measurable characteristics not mentioned here. Mature rat adipocytes have been analyzed by flow cytometry [14] and binding of lipoprotein lipase (LPL) antisera to cells from rat adipose depots has been quantitated by flow cytometry [15]. However, expression of developmentally regulated cell surface antigens by the preadipocyte cell subpopulation has not been analyzed using monoclonal antibodies and flow cytometry in any model system. The objective of this study was to characterize conditions for using monoclonal antibodies against preadipocyte cell surface antigens and fluorescence-activated flow cytometry to determine antigen expression by cultured S-V cells prior to lipid deposition. In order to further examine the utility of this technique, flow cytometric characterization of the regulation of AD-1 antigen expression by several anti-lipogenic growth factors was also performed.

MATERIALS AND METHODS

Cell Culture

Cells for culture were obtained by collagenase digestion of dorsal subcutaneous (sc) adipose tissues from one week-old pigs as described previously [16]. Cells in the S-V (non-floating) fraction were seeded at a density of 2.5×10^4 cells/cm^2 in 35 mm tissue culture dishes and cultured at 37°C in a humidified atmosphere containing 5% CO_2. The isolation procedure does not effectively dissociate capillaries, and cultures are essentially devoid of cells exhibiting characteristic endothelial cell morphology. All cultures were initiated in DME/F12 medium containing 10% fetal bovine serum (FBS) for the first 24 h. Cultures then either remained on FBS or were changed to medium containing 2% pig serum (PS) or PS and 10 nM dexamethasone (Dex). Treated cultures were supplemented at 24 h with 10 nM growth hormone (GH), 10 pM transforming growth factor-β (TGF-β), or 1 nM tumor necrosis factor-α (TNF-α) in Dex medium. Approximate doses of Dex [17], GH [18], TGF-β [19], and TNF-α [20] for inhibition of lipid deposition in S-V cultures have been demonstrated previously. After 3 additional days (4 days total), cultures were either stained with oil red-O [21] or processed for flow cytometry. Replicate cultures also were removed from their respective treatments, placed on PS-Dex medium and analyzed by flow cytometry three days after removal from treatment (7 days total).

Isolation of Cultured S-V Cells for Flow Cytometry

In preliminary experiments, several protocols including trypsinization, collagenase alone or in combination with hyaluronidase, and collagenase/hyaluronidase followed by EDTA treatment were used to produce single cell suspensions from cultures. Collagenase/hyaluronidase followed by EDTA treatment did not affect immunofluorescence using the AD-1 MAb. Moreover, greater than 98% of the cells harvested from cultures were viable single cells as indicated by trypan blue exclusion, and were capable of attachment and growth when subcultured (unpublished observation). Therefore, for isolation of single cells, cultures were rinsed three times with calcium- and magnesium-free (CMF) Hanks' balanced salt solution and treated with 0.5% collagenase/0.5% hyaluronidase in CMF-Hanks' at 37°C. After 10 min EDTA was added to yield a final concentration of 5 mM. After 10 additional minutes, liberated cells were collected and rinsed with DME/F12 medium and then placed on ice.

Flow Cytometry

All staining procedures were performed on ice. Aliquots containing $0.5–1 \times 10^6$ cultured cells from three dishes per treatment per experiment were incubated with isotype matched irrelevant IgG MAb or the AD-1 MAb (1/250 dilution of ascites fluids for 20 min), rinsed, and then incubated for an additional 20 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-

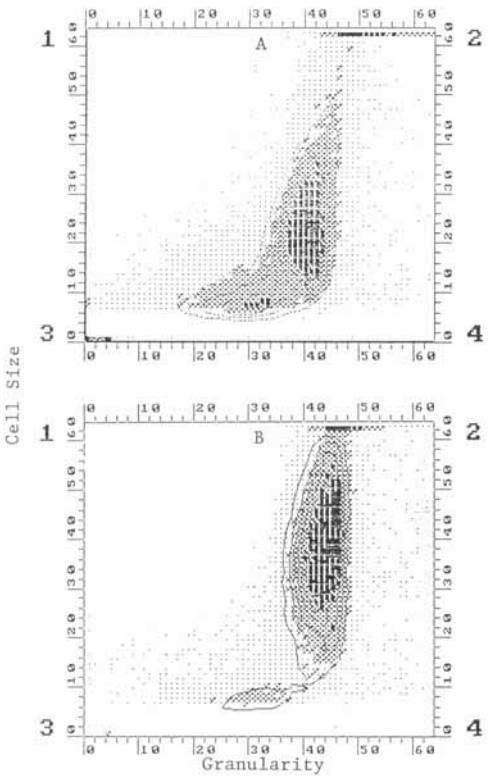


Fig. 1. Flow cytometric analysis of cultured stromal-vascular cells. Representative profiles demonstrating size versus granularity of cells grown for 3 days is PS-Dex (**A**) and PS-Dex supplemented with TGF-β (**B**).

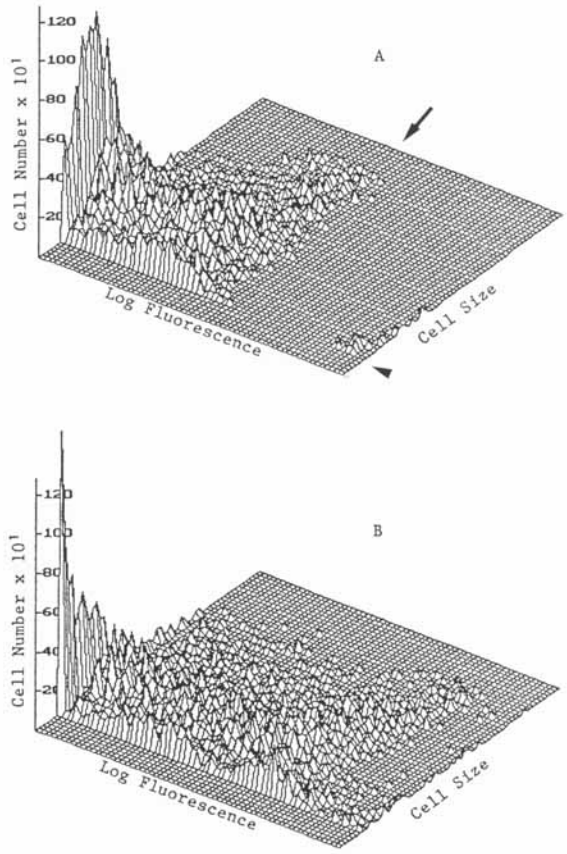


Fig. 2. Flow cytometric analysis of AD-1 antigen expression by cultured stromal-vascular cells. Representative three-dimensional profiles of cells grown for 3 days in PS-Dex and stained with control (**A**) or the AD-1 MAb (**B**). Arrow denotes cursor placement for control fluorescence levels. A small group of brightly fluorescent cells (arrowhead) are present in the control. For all samples, reported fluorescence values were corrected for values obtained from the replicate aliquot of cells from each treatment used as the control (see Fig. 3A,B).

mouse IgG (0.1 mg/ml, Sigma). Flow cytometry was performed using a Coulter Epics 753 argon laser flow cytometer. Stained suspensions were analyzed for fluorescence, granularity indicated by 90° light scatter and cell size indicated by forward angle light scatter. Maintenance of cells on ice during all staining steps proved crucial for preventing aggregation of cells and in allowing cells to assume a spherical morphology. Microscopic examination of stained suspensions just prior to cytometry indicated that the majority of cells containing detectable lipid droplets were removed by the multiple centrifugations. The percentage of cells in the final sample containing detectable lipid droplets ranged from 0% to 4% depending upon the culture conditions used. A sufficient number of cells for two analyses per treatment (AD-1 vs control) was obtained from one 35 mm culture. In this study, cells from three dishes were pooled for each treatment in each of four separate experiments. Light scatter gains and thresholds were set to eliminate debris. Cell size and granularity distribution patterns of cells grown in each medium formulation were examined in addition to fluorescence.

Statistics

In order to minimize differences between experiments, data were converted to percentage of antigen expression (or fat cell cluster formation) in controls before averaging. Data were subjected to analysis of variance using the general linear model procedures of the Statistical Analysis System [22]. Differences between treatment means were determined by least squares contrast [22].

RESULTS

Preliminary Characterization

Two representative cell size and granularity distribution pattern analyses are shown in Figure 1. Dot intensity is proportional to cell frequency. S-V cells were grown in several media

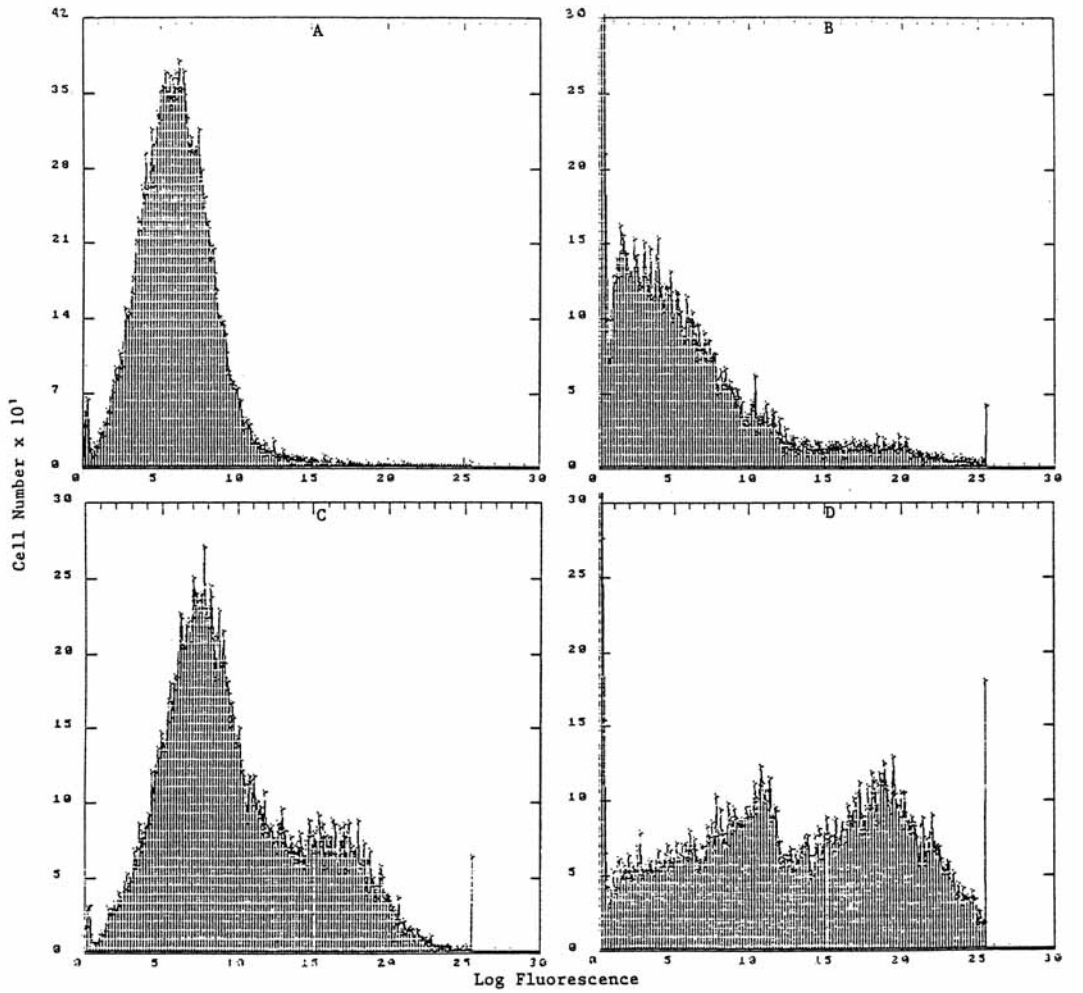
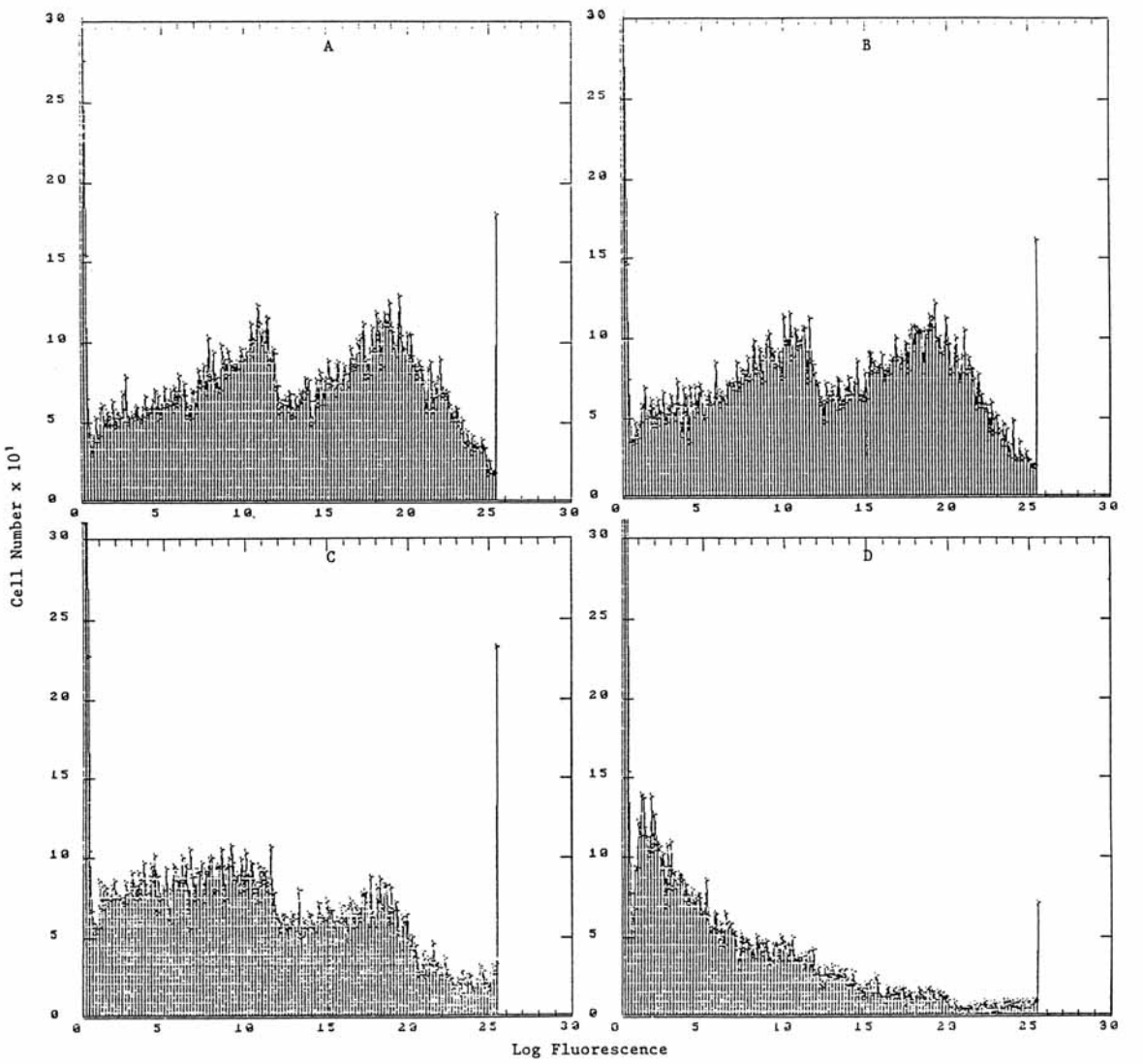


Fig. 3. Flow cytometric analysis of AD-1 antigen expression by cultured stromal-vascular cells. Replicate profiles (**A,B**) demonstrating log fluorescence of cells grown for 3 days in FBS and stained with control (A) or AD-1 (B). Profiles of cells grown in PS (**C**) and PS-Dex (**D**) are also shown. Replicate samples of PS and PS-Dex stained with control MAb are not shown, but exhibited the same distribution as the negative control shown in A.

formulations, including DME/F12 medium containing 10% FBS, 2% PS + 10 nM Dex (PS-Dex), and PS-Dex supplemented with GH, TNF-α, or TGF-β. In general, cells isolated from cultures maintained in each medium exhibited similar cell size and granularity distribution patterns (Fig. 1A), with the exception of cellular hypertrophy observed in medium supplemented with TGF-β (Fig. 1B). A representative three dimensional cell plot depicting size versus fluorescence distribution is shown in Figure 2B. Fluorescence values using the negative control MAb (Fig. 2A) in samples of cells grown in each condition resulted in approximately 4% background fluorescence based on the settings depicted by arrows in Figure 2A. In all analyses, fluorescence values were corrected for values obtained in the replicate control sample. Examination of cells obtained by sorting material collected from the positive subpopulation indicated that fluorescence was detectable microscopically on >95% of the cells measured as fluorescence-positive.

Effect of Basal Medium on AD-1 Antigen Expression

The proportion of cells expressing detectable levels of the AD-1 antigen was dependent upon medium composition (Fig. 3). After 4 days of culture (3 days on treatment), more S-V cells expressed the antigen in PS-Dex cultures (Fig. 3D) than PS or FBS cultures. Since the proportion of AD-1 positive cells continued to increase up to 7 days of PS-DEX culture (not shown) whereas antigen expression in FBS- and PS-



supplemented cultures diminished, PS-Dex supplementation was used as differentiation medium in all further experiments.

Regulation of Antigen Expression by GH, TGF-β, and TNF-α

Stromal-vascular cells were cultured for three days in differentiation medium alone or medium supplemented with GH, TNF-α, or TGF-β. Cultured cells were either stained for lipid or stained with the AD-1 MAb and analyzed by flow cytometry after three days on treatment (Fig. 4) and upon three additional days of culture, after removal from treatment (Fig. 5). Data are summarized in Figures 6 and 7. GH, TNF-α, and TGF-β each inhibited lipid deposition (day 4, $P < 0.001$, Fig. 6), but exhibited different effects on AD-1 antigen expression. GH had no effect on AD-1 antigen expression (day 4) whereas TNF-α and TGF-β each reduced (day 4, $P < 0.001$) the proportion of cells expressing the antigen as compared to differentiation medium alone (Figs. 4, 7). Fat cell cluster formation increased significantly after removal from TNF-α and TGF-β treatment (Fig. 6) but did not reach control values, while inhibition of cluster formation was not reversed after removal of GH in GH-treated cultures. AD-1 antigen expression increased upon removal of TNF-α and TGF-β (day 7, Figs. 5, 7), and AD-1 immunoreactivity continued to

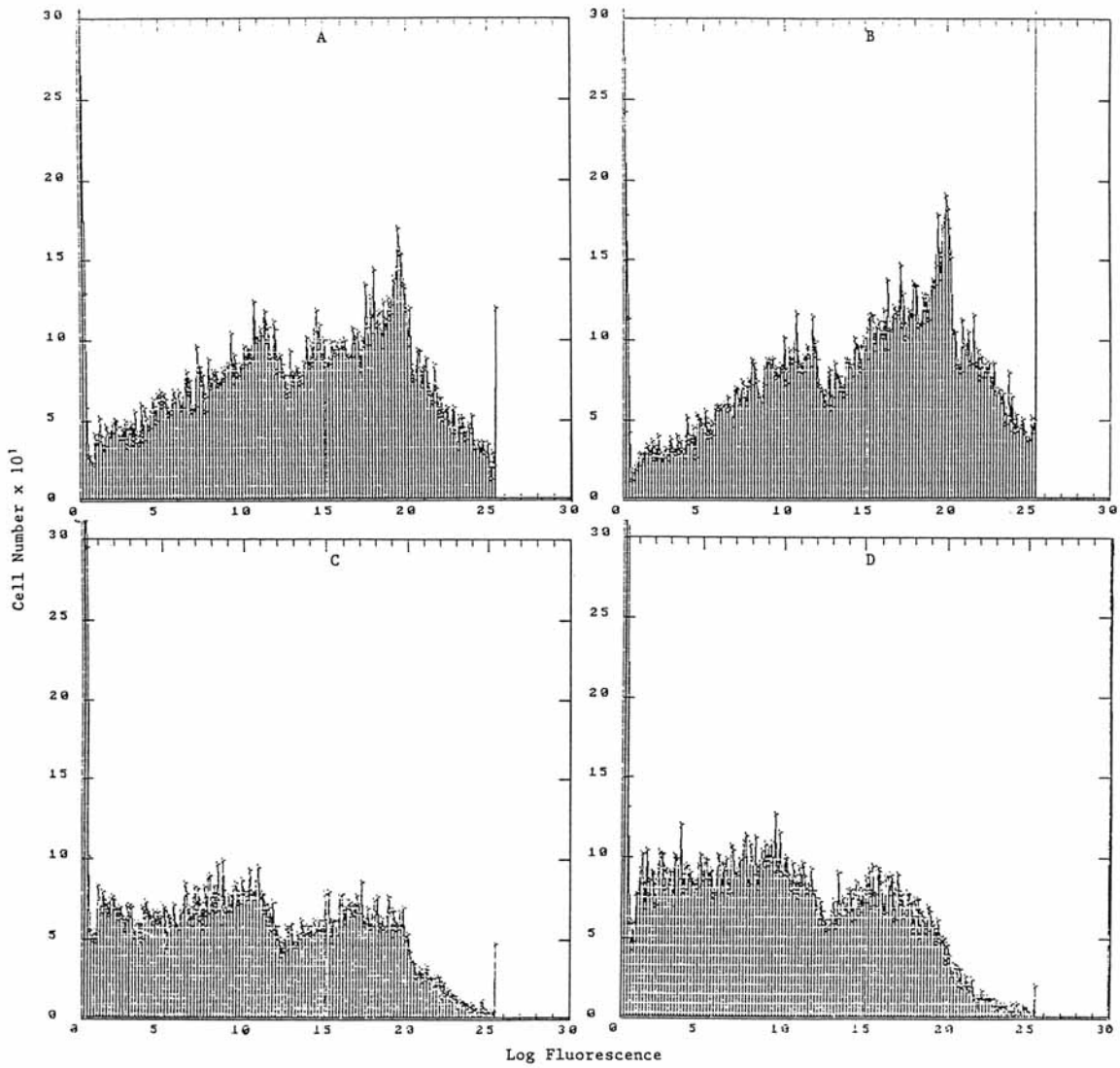


Fig. 5. Flow cytometric analysis of AD-1 antigen expression by cultured stromal-vascular cells. Cells were treated as in Figure 4, removed from treatment and cultured for 3 additional days in PS-Dex alone. Representative profiles of cells stained with AD-1 MAb after 6 days in PS-Dex (**A**) or 3 days after removal from GH (**B**), TNF-α (**C**), or TGF-β (**D**).

increase in GH-treated cultures independent of inhibition of lipid deposition.

DISCUSSION

We have described a methodological approach for using flow cytometry to analyze a subpopulation of S-V cells defined by a MAb designated AD-1. Based on previous studies examining localization of cells expressing the AD-1 antigen in tissues, it seems reasonable that the cell subpopulation defined by AD-1 includes the preadipocyte [11,12]. The exact proportion of adipogenic precursors in S-V cultures can only be determined by the number of cells that differentiate, and the proportion of cells that differentiate is dependent at least in part upon the differentiation medium used. In the present study, Dex enhanced AD-1 antigen expression over PS alone (Fig. 5) and cultures of preadipocytes grown in PS supplemented with Dex systematically produced more fat cell clusters than PS alone [23,24]. The cytometric profiles comparing Dex to PS (Fig. 3) may indicate that more precursor cells differentiated in DEX treated cultures within the normal time course of culture. On the other hand, Dex may be triggering additional cells toward becoming adipogenic cells. In a previous study, the proportion of cells in S-V

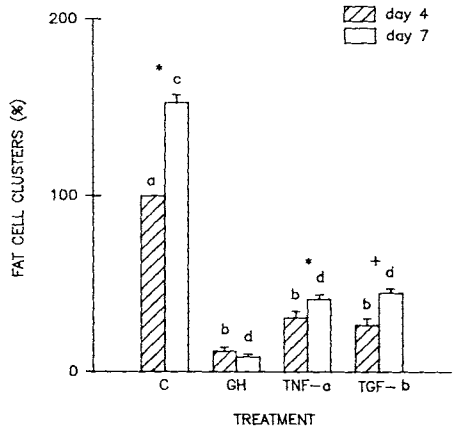


Fig. 6. Lipid deposition in cultures. Cells were stained for lipid with oil red-O. Cultures were maintained in PS-Dex for the entire culture period (C) or treated for 3 days with growth hormone (GH), TNF-α or TGF-β for 3 days (day 4) followed by 3 additional days of culture on PS-Dex alone (day 7). Summary of results obtained in four separate experiments. Means are based on differentiation medium alone, as 100%. Columns with different letters within a time period differ from control ($P < 0.001$). Columns with * differ over time within treatment ($P < 0.001$), or + ($P < 0.003$).

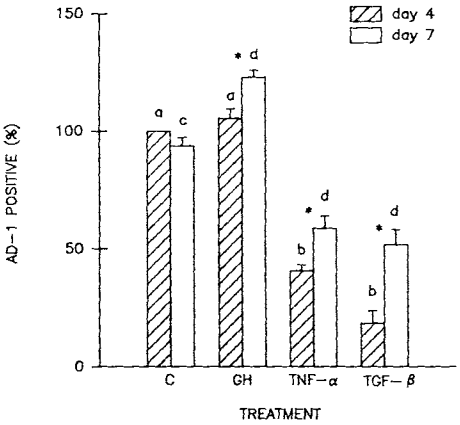


Fig. 7. Flow cytometric analysis of cultured stromal-vascular cells. Summary of results obtained in four separate experiments. Means are based of differentiation medium alone as 100%. Columns with different letters within a time period differ from control ($P < 0.001$). Columns with * differ over time within treatment ($P < 0.001$).

cultures expressing the AD-1 antigen was initially high (approximately 45% of the total cells from newborn pig) when the cells were maintained in medium supplemented with 10% FBS alone [10]. However, antigen expression decreased in cultures if maintained in medium supplemented with FBS alone and such cultures exhibited relatively low levels of differentiation.

Direct correlation of AD-1 immunoreactivity and lipogenic enzyme expression by cell subsets was not within the scope of this study. However, AD-1 antigen expression was not related to lipid deposition in all instances. For example, GH, TNF-α and TGF-β are all potent inhibitors of lipid deposition. Three day exposure of S-V cultures to each factor blocked lipogenesis (Fig. 6) while only TNF-α and TGF-β affected AD-1 antigen expression. After removal from treatment, lipid deposition in TNF-α and TGF-β treated cultures resumed whereas lipid deposition in GH-treated cultures remained inhibited. The exact reason for this maintenance of inhibition of lipid deposition by exposure to GH in the presence of DEX is not clear.

Several groups have attempted to isolate homogenous populations of preadipocytes from primary adipose tissues using buoyant centrifugation [3,25,26]. However, this technique separates cells primarily based upon triglyceride content and therefore after the onset of overt differentiation. Similarly, cells with detectable levels of surface-bound LPL [15] are likely committed to differentiate. On the other hand, AD-1 positive cells have been detected in tissues long before adipocyte differentiation [12]. During the preliminary characterization of flow cytometry using cultured S-V cells, several different trials were attempted for subculturing the sorted AD-1 positive cells (unpublished). When AD-1 positive cells were subcultured using medium supplemented with 2% pig serum and Dex, lipid deposition was detectable in the vast majority of the AD-1 positive cells, but the cells failed to remain attached to the culture dish. If the sorted cells were first subcultured for 24 h in FBS and then changed to medium supplemented with 2% pig serum without Dex, the cells attached and >95% of the cells stained positive for lipid using oil red-O by 48 h on PS. In contrast, less than 5% of the cells in the antigen negative subpopulation stained for lipid up to 72 h after the onset of subculture. At present, the primary difficulty with using flow cytometry for establishing subcultures appears to be in obtaining sufficient numbers of single cells within a reasonable amount of time since unsorted S-V preparations tend to aggregate during extended sorting experiments. Experiments in progress are aimed at improving efficiency of cell sorting by lowering sorting temperatures and changing the formulation of media used for sorting. By using techniques such as flow cytometry it may be possible to study events regulating entry of cells into the adipogenic lineage prior to overt acquisition of

the adipogenic phenotype, and to establish which cell lines may be useful as model systems for studying events related to determination as opposed to differentiation.

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