EFFECT OF DEFIBROTIDE ON CULTURED ENDOTHELIAL CELL CYCLE PROGRESSION ANALYSED BY FLOW CYTOMETRY

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Ç. Erşahin, M.D.** / S. Bilsel, PhD** / A. Yalçıner M.D.*** / K. Emerk, PhD.***

* *Profesor, Department of Biochemistry, Faculty of Medicine, Marmara University, Istanbul, Türkiye.*
** *Assistant Professor, Department of Biochemistry, Faculty of Medicine, Marmara University, Istanbul, Türkiye.*
*** *Biochemistry Specialist, Düzen Laboratories, Istanbul, Türkiye.*
**** *Research Assistant, Department of Biochemistry, Faculty of Medicine, Marmara University, Istanbul, Türkiye.*

SUMMARY

Defibrotide (D) is an antithrombotic and profibrinolytic drug which is prepared from mammalian lung DNA. Studies using radiolabeled defibrotide demonstrated that considerable amount of the drug goes to the nuclei of cultured endothelial cells. Since the drug goes to the nuclei, it may exert its effects by induction of gene expression of certain proteins and/or by influencing cell cycle progression. In this study, we used flow cytometric techniques to estimate the effects of defibrotide on cell cycle parameters of cultured endothelial cells. No statistical difference in the fraction of cells at G_1, S, G_2 +M phases was observed between control cells and the cells treated with defibrotide both at different times and confluency levels.

Key Words: Endothelium, flow cytometry, Defibrotide

INTRODUCTION

Defibrotide is a deoxyribonucleic acid derivative extracted from mammalian tissues. It is the sodium salt of single-strand polydeoxyribonucleotide of mean molecular weight 15 to 30 kD with defined ratio of purine to pyrimidine bases of >0.85 (1). The drug has antithrombotic and profibrinolytic activity due to its ability to modulate endothelial function. Defibrotide increases prostaglandin I_2 and E_2 levels, tissue plasminogen activator secretions and decreases plasminogen activator inhibitor production of endothelial cells (2,3). It has also been shown that protein content of cultured human umbilical vein endothelial cells (HUVEC) increased following incubation with defibrotide and defibrotide enhanced the proliferation of cells (4). Studies using radiolabeled defibrotide demonstrated that the major binding site of the drug is the plasma membrane but also considerable amount is found in the nuclei (5). Since the drug appears to go to the nuclei, it may exert its effect by induction of gene expression of certain proteins and/or by influencing cell cycle progressions. In this study flow cytometric techniques were used to estimate the effects of defibrotide on cell cycle parametres. Cultured HUVEC at different confluency levels from day 2 to 7 incubated with medium and medium supplemented with defibrotide (100 µg/ml) were compared by determining the fraction of cells in the G_1, S and G_2 + M phases at certain intervals.

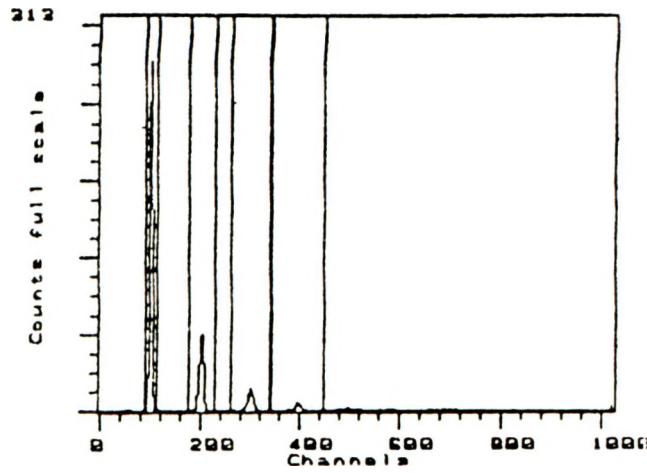
MATERIALS AND METHODS

Isolation and culture of endothelial cells:
Human umbilical vein endothelial cells were isolated by enzyme digestion with (0,1 % collagenase) according to the method of Jaffe with some modifications (4-6). Half of the cells were suspended in medium 199 supplemented with fetal calf serum (20 %) and antibiotics, while the other half suspended in the same medium containing 100 µg/ml defibrotide. Cells were seeded into plastic wells of 1.4 mm. diameter (NUNC) with equal density (2.5 x 10^4 / cm2). The cultures were incubated at 37°C in a CO2 incubator (5% CO2, 95% air) and the culture medium was changed every 2 days. Cells were identified by the presence of Von Willebrand factor in their medium and by their characteristic cabble-stone morphology under light microscope.

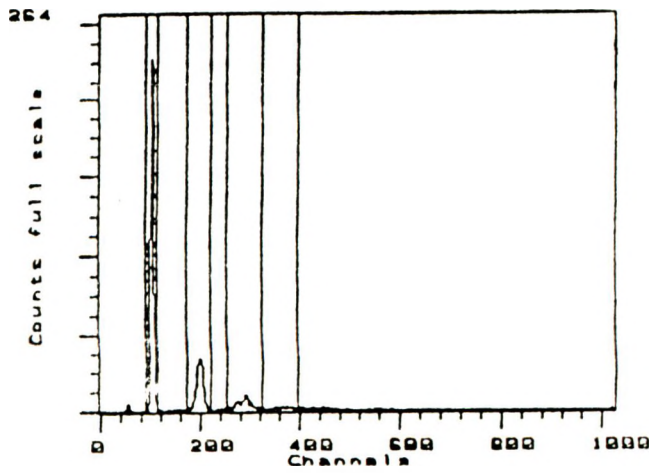
Cell Cycle Analysis By Flow Cytometry:
Growth response of the cells to defibrotide compared to control cells was analyzed by flow cytometry (Facscan/Becton Dickenson), where a cell suspension is forced through a nozzle at a rate of several thousand cells per second, and an optical measurement is made and recorded for each individual cell as it briefly passes a tiny aperture. Starting on the 2nd day of culturing till 7 th day, cells were harvested by EDTA solution, washed with buffer and treated with pepsin and 0.02ng/ml RNAse and were stained by incubation in solution containing 0.05 ng/ml propidium iodide (PI). Tubes were vortexed for 10 seconds and allowed in incubation at least 20 minutes on ice. PI stained nuclei were analyzed by exciting the dye with a laser beam and measuring the emitted fluorescence of single cells as they flow in a single file at high rates. Data were analysed by using CELL FIT software version 2. The DNA content and the fraction of cells in G_1, S and G_2 + M phases were evaluated. Those cells with the least amount of DNA were in G_1, those with double this amount were in G_2 or M, while cells in S had intermediate amounts.

Facscan was standardized by chicken erythrocyte nuclei (CEN) and coefficient of variation was around 3% for the areas, and 5% for the heights (Fig.1).

Endothelial cells isolated directly from umbilical cord vein was used as control and the gates were chosen in order to include singlet transmissions (Fig.2), dot plot diagram and histograms were compared for each set of experiment to check the standardization of the procedure so that cultured cells can be compared with respect to proliferation status.

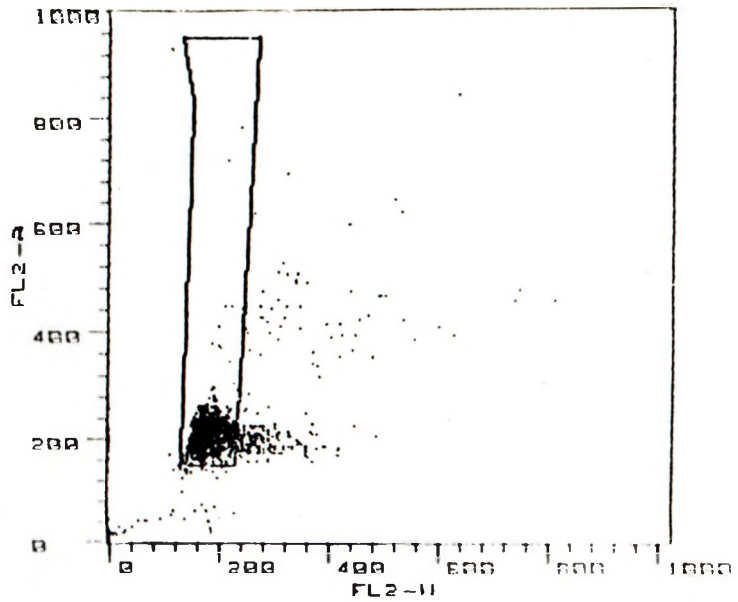


CEN in FL2-A Histogram



CEN in FL2-H Histogram

Fig I. Typical fluorescence area and height histograms of PI stained chicken erythrocyte nuclei (CEN). CEN are used tc standardize FACScan for DNA analysis. The CEN preparation contains singlet nuclei, plus doublets, triplets and some larger aggregates which gives four or more reference peaks in the DNA stain histograms. The major peak represents single nuclei. The next three peaks have mean channel values approximately 2,3,4 times greater than the single nuclei peak. The coefficient of variation of the singlet peak must be less than 3% for a nuclei sample stained.



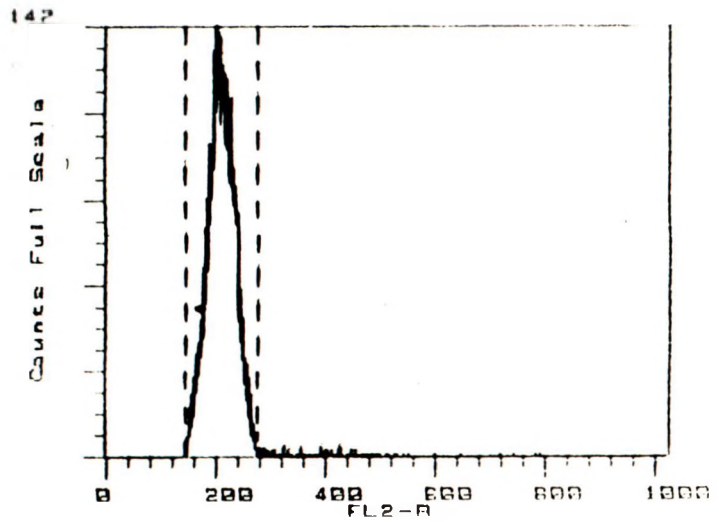


Fig II. Typical correlated light scatter and fluorescence histograms for endothelial cells isolated from human umbilical cord vein. The histogram (b) shows 98.07% of the cells are in G_0/G_1 phase. Data has been gated as shown in (a). The mean, the median, the mode of the curve are 211, 211, 201 respectively.

RESULTS AND DISCUSSION

Growth of cell populations can be analyzed by considering individual cells to progress sequentially through a series of phases; mitosis (M), gap 1 (G_1), synthesis of DNA (S), and finally gap 2 (G_2). For the investigation of effect of defibrotide on cell cycle progression, endothelial cells isolated from umbilical cord veins were pooled and cultured in multiwelled plates with equal density with medium containing D (100µg/ml) or not. Starting from the second day of culturing till the seventh day, cells were harvested and the fraction of cells in G_1, S, G_2+M phases were determined.

Comparison of the fraction of cells in certain phases at certain time intervals showed no significant difference between control cells and cells incubated with medium supplemented with D. Also, when the cells were grouped according to degree of confluency (determined by the number of cells in each well, instead of day of culturing), no statistical difference was observed between control cells and cells treated with defibrotide (Table I). Mann-Whitney test was used to compare the groups, and the two-tailed P value obtained varied between 0,2544-0,4857. Total of 90 cell cultures were evaluated.

Since defibrotide goes to the nucluei it may exert its effects by induction of gene expression of certain proteins and/or by influencing cell cycle progressions. Although it has been reported that anastomised veins show remarkable growth characteristics suggesting some type of stimulation of endothelial cell growth (7), we could not observe any significant change in the length of different phases of cell cycle in our study. According to our results the effect of defibrotide does not involve observable changes in the length of different phases of cell cycle. This may be due to use of non-synchronized cells in our experiment. The proliferative effect of the drug remains to be explored. But the reported increase in protein concentration in HUVEC may be explained by the effect of drug on gene expression of certain proteins.

Table I: Estimated mean value of fraction of cells in G_1 phase (m) in culture at indicated confluency levels; statistical analysis was performed by Mann-Whitney test.

Confluency	0-50%	50-65%	65-80%	80-95%	95-100%
Defibrotide	m = 36 (n = 10)	m = 68.5 (n = 10)	m = 71 (n = 10)	m = 76 (n = 9)	m = 88 (n = 10)
Control	m = 52 (n = 10)	m = 78 (n = 8)	m = 78 (n = 7)	m = 70.5 (n = 8)	m = 63 (n = 10)
P value	0.3176	0.3824	0.2721	0.4857	0.2544

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