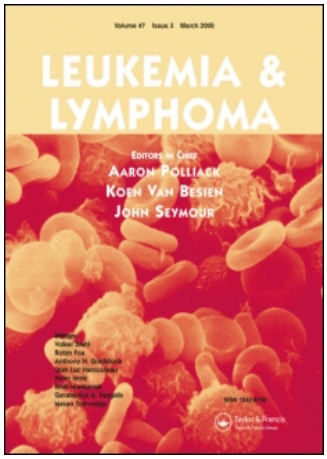

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Separate cell culture conditions to promote proliferation or quiescent cell survival in chronic lymphocytic leukemia

Shaun Willimott [a]; Maria Baou [a]; Sarah Huf [a]; Simon D. Wagner [a]
[a] Division of Investigative Sciences, Department of Haematology, Imperial College London, Hammersmith Hospital, London, UK

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LETTER TO THE EDITOR

Separate cell culture conditions to promote proliferation or quiescent cell survival in chronic lymphocytic leukemia

SHAUN WILLIMOTT, MARIA BAOU, SARAH HUF, & SIMON D. WAGNER

Division of Investigative Sciences, Department of Haematology, Imperial College London, Hammersmith Hospital, London W12 0NN, UK

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Chronic lymphocytic leukemia cells circulating in the peripheral blood are in the resting phase (G_0/G_1) of the cell cycle, yet, over time, leukaemic cells accumulate. Recently, proliferation rates in vivo have been shown to be substantial, with the most clinically aggressive cases having the highest proliferation rates [1]. The sites where CLL cell division takes place are proliferation centres found in lymph nodes [2].

The characteristics and proliferative capacity of leukaemic cells within the lymph nodes or bone marrow are quite different to those of cells in the peripheral blood, and simple culture system that can separately maintain quiescent (representing peripheral blood CLL cells) and proliferating CLL cells will be useful in analysing the biology of CLL and testing the effects of drugs and new biological agents.

Contact with a specialized stromal cell layer (bone marrow derived stromal cells) has been demonstrated to maintain CLL cell viability [3] and prevent spontaneous apoptosis [4]. Others have shown similar effects with in vitro derived nurse-like cells [5]. Both of these cell types are demanding to produce and difficult to maintain.

Proliferation of CLL cells in vitro has been produced in various systems. CD40 ligand (CD154) is a T-cell surface molecule that is essential for B-cell activation and the formation of germinal centres by normal B-cells. It has also been used extensively in CLL research [6 – 9]. Modest proliferation of CLL cells in vitro can be produced by signalling through CD40 but this is greatly enhanced by co-activation with IL-4 [7]. This effect is specific to IL-4 and the addition of IL-2 to CD40 signalling does not produce significant enhancement of proliferation [7]. CD154 is expressed on T-cells within lymph node proliferation centres, and is physiologically relevant for CLL cells [2]. The combination of soluble CD40 with IL-4 has also been used to culture CLL cells [10] and produce proliferation. Autologous primary T-cells, activated by anti-CD3, are an alternative means of providing signals to drive CLL cell proliferation [9]. In a conceptually entirely different approach, CLL cells have been driven into cell-cycle by CpG-oligonucleotide combined with IL-2 [11]. Previous work has not specifically addressed the issue of how to compare biological and drug responses of viable but quiescent and proliferating CLL cells.

We first compared survival and proliferation of CLL cells cultured on mouse fibroblast L-cells (NT-L) and a specialized stromal cell layer (human bone marrow derived mesenchymal stem cells (MSC) gift of Professor Francesco Dazzi, London) [Figure 1(a)]. Both cell types were irradiated before culture with CLL cells. MSC were used because they are important for haemopoietic cells [12,13] and can specifically support the growth of B-cell lymphomas. Blood samples were obtained from CLL patients with a diagnosis confirmed using the NCI Working Group definition. Patients in this cohort were from all clinical stages and had not been treated for at least 12 weeks prior to the study. Patient age ranged from 44 – 85 years (mean, 69 years). Patients with white cell counts $> 50 \times 10^9\, l^{-1}$ were selected for this study. CLL cells were isolated from heparinized venous blood by density gradient centrifugation and

Correspondence: Simon D. Wagner, Department of Haematology, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN. Tel: +0208-383-2164. Fax: +0208-740-9679. E-mail: simon.wagner@imperial.ac.uk

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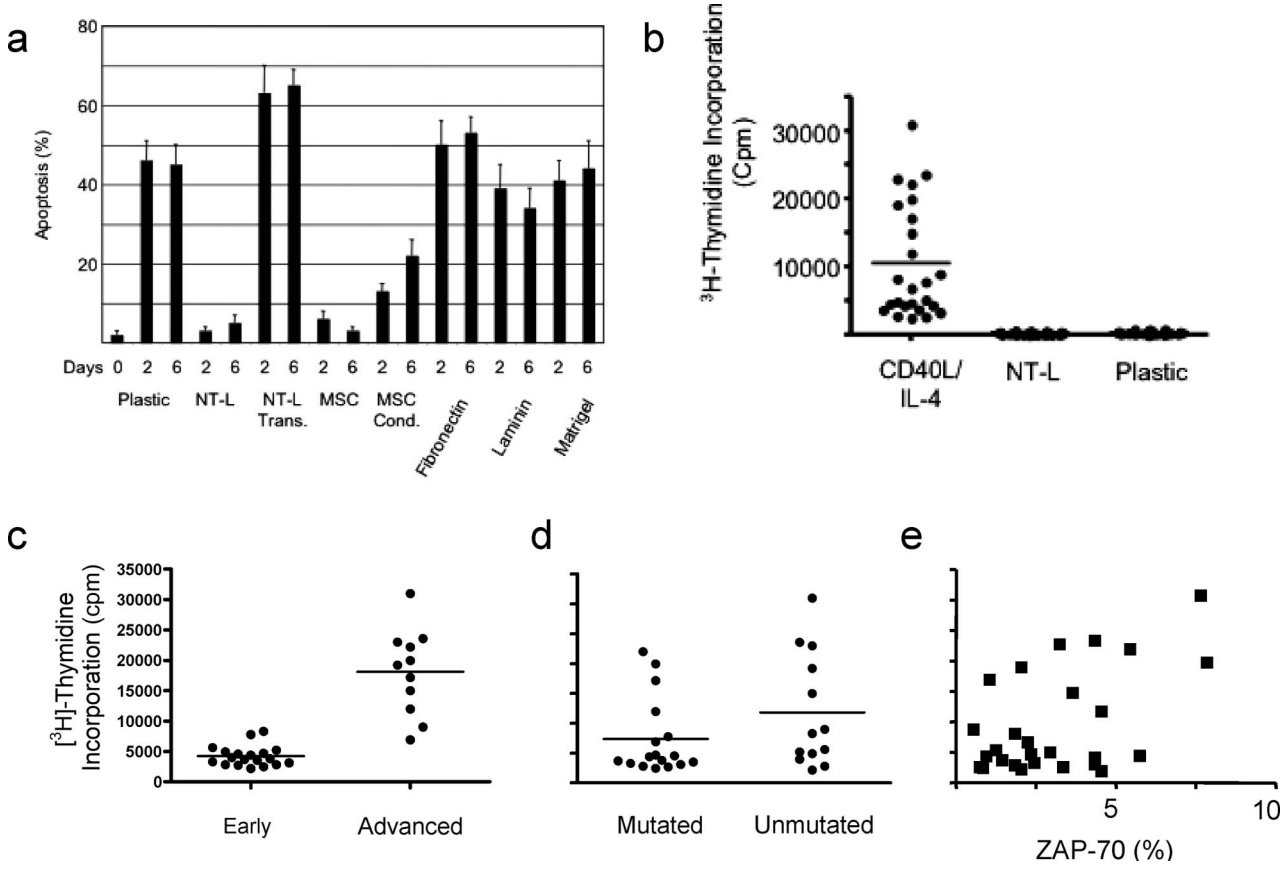

Figure 1. CLL cell apoptosis and proliferation under different culture conditions. CLL cells were re-suspended at 3×10^6 cells/ml in RPMI1640 medium (Cambrex, UK) supplemented with 10% FBS (Cambrex, UK), non-essential amino acids (Invitrogen, UK), antibiotics (Invitrogen, UK), and Hepes buffer (Cambrex, UK). Isolated CLL cells were then cultured in 6-well plates for 0–6 days. We used 30-Gy irradiated mouse fibroblast L-cells or L-cells transfected with CD154 (gift of Prof. J. Gordon, Birmingham), together with IL-4 (R&D Systems) at 20 ng/ml, as stromal cell layers to investigate the variability in proliferative response in individual cases of CLL. (a) Apoptosis, measured by staining for annexin V binding (BD Biosciences), after culture on tissue culture plastic, mouse fibroblast L-cells, mesenchymal stem cells, and basement membrane components. The results presented are means ± SEM for six patients. Apoptosis was measured at 0, 2, and 6 days. (b) Proliferation was measured by [^{3}H]-thymidine incorporation in CLL cells cultured in the CD154/IL-4 system and compared with CLL cell cultures on NT-L cells and plastic. Only the CD154/IL-4 system produces significant proliferation. (c) Association between clinical stage and proliferation ([^{3}H]-thymidine incorporation). Advanced clinical stage (Binet stage B or C, or progressive stage A) is associated with significantly higher rates of proliferation ($p < 0.001$, Mann–Whitney U Test). (d) Association between immunoglobulin gene mutational status and [^{3}H]-thymidine incorporation. There is no significant difference between the two groups (Mann–Whitney U Test). (e) Association between ZAP-70 expression in CLL cells measured by flow cytometry and [^{3}H]-thymidine incorporation. Linear regression shows a non-significant association ($r^2 = 0.266$).

cultured immediately. After 6 days culture on tissue culture plastic annexin V binding cells accounted for between 40% and 50% of all cells, but NT-L and MSC both markedly improved survival. MSC secrete growth factors [13] and MSC conditioned medium (MSC Cond) also promoted CLL cell survival. By contrast, culture of CLL cells in transwells above a layer of NT-L reduced survival to levels observed on plastic demonstrating that any growth factors secreted by NT-L cells are not sufficient to promote CLL cell survival, whereas those secreted by MSC can improve CLL cell viability. Basement membrane components (laminin, fibronectin and Matrigel), which are elaborated by stromal cells in vivo, did not promote survival. We conclude that cell–cell contact and growth factors have redundant roles in promoting CLL cell survival in vitro, and that cell–cell contact in the absence of specific factors is highly effective in promoting survival.

We next investigated whether improved survival mediated by NT-L cells was associated with proliferation. [^{3}H]-thymidine incorporation (after 5 days in culture) demonstrates that the observed changes in survival do not translate into an increase in proliferation [Figure 1(b)].

L-cells stably transfected with CD154 (gift of Professor John Gordon, Birmingham) combined with IL-4 (CD154/IL-4 system) produced proliferation in all patients tested, as compared to NT-L cells, but there was a wide range of responses. Clinically advanced stage disease is often associated with rapid lymphocyte doubling time. High [^{3}H]-thymidine

incorporation is significantly higher ($p < 0.001$, Mann–Whitney U test) with advanced clinical stage [Figure 1(c)], suggesting that proliferation driven by CD154/IL-4 stimulation in vitro reflects proliferative capacity in vivo. Cell cycle analysis using propidium iodide staining showed that 16.4 ± 3.2% (mean ± SEM) of CLL cells in the CD154/IL-4 system were in S-phase or G_2M of the cell cycle, but on NT-L or plastic, the fractions were 3.4 ± 1% and 2.7 ± 1%, respectively.

To demonstrate the functional importance of the different culture conditions, CLL cells were incubated with either fludarabine or staurosporine [Figures 2(a) and 2(b)]. Whilst the effects of staurosporine were

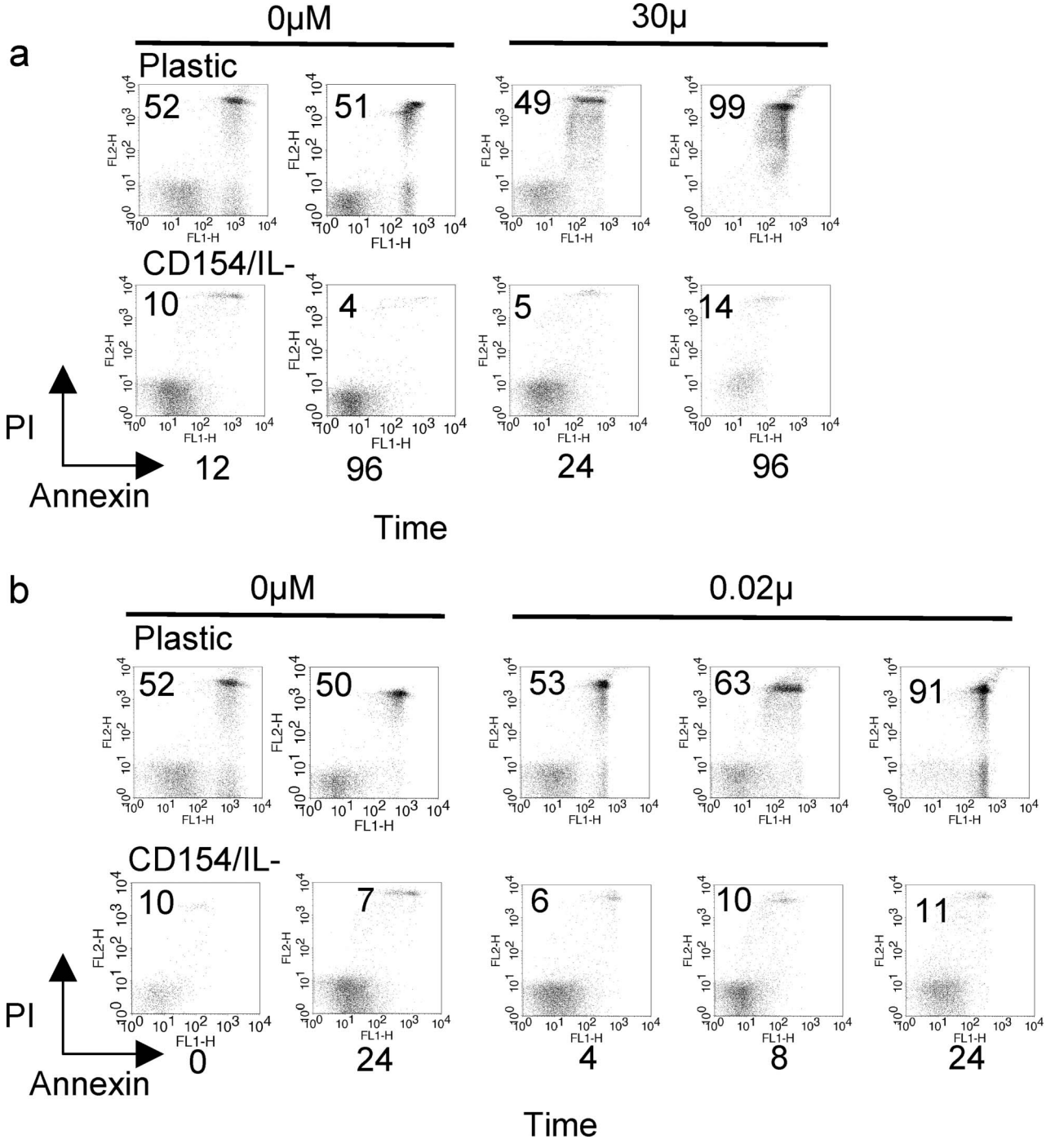

Figure 2. Comparison of the effects of culture on plastic or in the CD154/IL-4 system on protection from drug induced apoptosis. (a) Apoptosis due to fludarabine (30 μM; Schering). Apoptosis was measured at 12, 24, and 96 h after cells were placed in culture. The percentage of annexin V binding cells is given in the top left hand corner of the dot-plot. (b) Apoptosis due to staurosporine (0.02 μM; Sigma–Aldrich). Apoptosis was measured at 0, 4, 8, and 24 h after leukaemic cells were placed in culture.

much more rapid than those of fludarabine, the effects of both drugs in inducing apoptosis were reduced on culture in the CD154-L cell/IL-4 system. There was no correlation between the degree of apoptosis and [^{3}H]-thymidine incorporation.

To simplify the culture system, we did not specifically purify leukaemic B-cells from peripheral blood mononuclear cells, although in all cases CLL cells accounted for $>90\%$ of this population. As described, we found significant differences in CLL cell apoptosis and proliferation between the culture systems. Contaminating cells (T-cells, monocytes or nurse-like cells) were present in each of the three culture conditions tested and, therefore, are unlikely to have had a major influence on the results, although we cannot exclude minor effects.

CLL cells are often studied in vitro by short-term cultures on tissue culture plastic. In this context, the leukaemic cells remain in G_0/G_1 of the cell cycle and die rapidly by apoptosis. In vitro systems are utilised to test the effects of drugs as well as analyse the biology of the disease, but high spontaneous apoptosis will tend to obscure drug effects and possibly biological effects. For many purposes, the use of mouse fibroblast L-cells will be sufficient to maintain CLL cell viability.

The novelty of this report is in the definition of a culture system allowing the direct comparison of quiescent viable CLL cells with proliferating cells. The distinction between signals required for survival and proliferation has not previously been appreciated, but is clearly demonstrated here. We have shown that this is important for the analysis of drug responses and it is likely to be important for the investigation of CLL biology.

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