(Trachootham *et al.*, 2008). It is worth noting that recent studies are targeting the molecular mechanisms that control the redox environment in leukemia cells, made up from the production of ROS and the expression and activity of antioxidant enzymes. It has been demonstrated that leukemia cells gain proliferative and survival advantages by manipulating this system. Therefore, ROS production by the mitochondrial electron transport chain, NADPH oxidase, xanthine oxidoreductase, and cytochrome P450, have all been targeted to promote leukemia cell death (Irwin *et al.*, 2013). In this Chapter, the results suggest that CD38 might also serve as a possible target to regulate the redox system specifically in $CD38^+$ leukemia cells through controlling its role in ROS production.

The data presented in this Chapter reveal novel findings concerning the effects of NAD depletion on cell physiology. As discussed above, NAD depletion during HL60 differentiation caused an enhancement of lipid peroxidation, and relative depletion of released lactate and total glutathione levels, while the NAD^+:NADH ratio remained relatively constant. These data raise the possibility that lowered NAD levels might have effects on NAD-dependent processes such as glycolysis, depending on the availability of NAD^+ (Kristian *et al.*, 2011). An insufficient supply of NAD^+ limits cellular energy production (Wilhelm and Hirrlinger, 2012), and it might also affect cell survival and cause cell death (as in differentiated HL60 cells). However, the consequences of lowered NAD levels via CD38 expression in leukemia cells might promote anti-apoptotic effects, as shown in Figure 4.10.

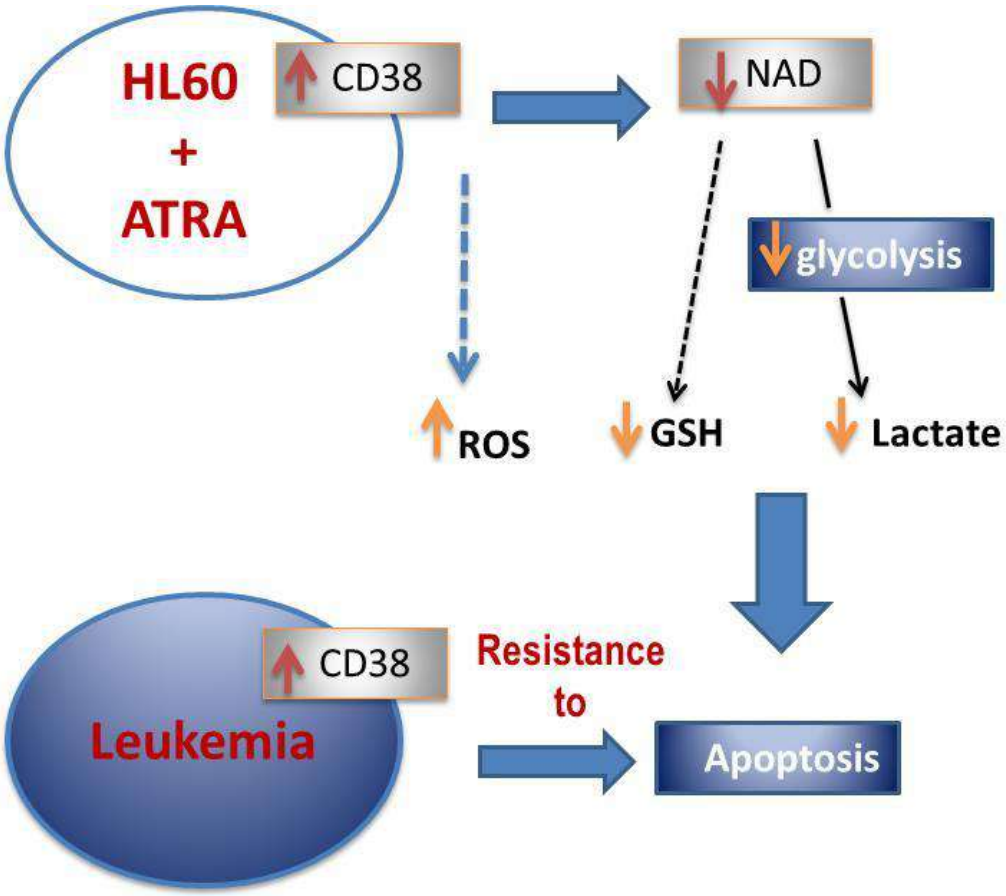


Figure 4.10 The consequences of lowered intracellular NAD levels on cell metabolism. This diagram describes the role of CD38 expression as a determinant of NAD- mediated cell survival, leading to either apoptosis in differentiated HL60 cells or anti-apoptotic effects in $CD38^{+}$ leukemia cells.

CHAPTER 5

REGULATION OF CD38 EXPRESSION

5.1 Introduction

The involvement of CD38 expression in various types of cells and in several diseases makes it a possible therapeutic target, especially in leukaemia. Thus, transcriptional regulation of CD38 has been extensively studied. Cytokines and hormones are two major groups of signalling molecules implicated in the regulation of CD38 expression. In CLL, studies have shown that interferons (IFN-α, -β and -γ), IL-2 and IL-4 increase CD38 mRNA expression (Bauvois *et al.,* 1999; Deaglio *et al,* 2003; Levesque *et al.*, 2006). Furthermore, the effects of tumour necrosis factor-α (TNF-α), IFN-γ, IL-1β and the Th2 cytokine, IL-13, on the increase of CD38 mRNA have also been studied in human airway smooth muscle (HASM) cells (Deshpande *et al.,* 2004). Further studies suggest a transcriptional upregulation of CD38 by TNF-α in myometrial cells (Barata *et al.,* 2004) and macrophages (Iqbal, and Zaidi, 2007). Moreover, in human monocytes and the derived lines U937 and THP-1, the study of Musso *et al.* (2001) found that IFN-γ and IL-2 increased CD38 expression but that lipopolysaccharide (LPS), TNF-α and GM-CSF had no detectable effects. However, further studies showed that LPS increased CD38 mRNA expression in J774 macrophage cells (Lee *et al.*, 2012a).

The effects of hormones on CD38 expression have been studied in myometrial tissue (Dogan *et al.,* 2002; 2006). In ovariectomized rats, administration of estradiol-17β caused a significant induction of CD38 expression in the myometrium (Dogan *et al.,* 2004). However, glucocorticoids (a class of steroid hormones) have been found to inhibit CD38 expression as shown in HASM cells (Kang *et al.,* 2006; 2008).

Transcriptional regulation of CD38 has been also investigated in the HL60 cell line during differentiation with various agents. It was observed that differentiation of HL60 to granulocytes induced by isonicotinic acid, led to CD38 expression (Iwata *et al.,* 2003). Moreover, 1α,25-dihydroxy vitamin $D_{3,}$ which is an inducer of differentiation of HL60 towards monocyte-like cells, has also been shown to induce CD38 expression (Stoeckler *et al.,* 1996). Importantly, the differentiation of HL60 into granulocytic cells using ATRA is accompanied by the induction of CD38 expression (Drach *et al.,* 1993), while DMSO has no effect on CD38 expression (Iwata *et al.,* 2003; Guida *et al.,* 2004). It was suggested that ATRA-induced CD38 expression is mediated by direct transcriptional regulation via activation of a RAR/RXR heterodimer interacting with a retinoic acid response element located in the first intron of the CD38 gene (Mehta *et al.,* 1997). In undifferentiated-HL60 cells, a mitochondrial NADH dehydrogenase inhibitor, rotenone, was also shown to induce CD38 expression (Matsunaga *et al.,* 1996). Importantly, the finding of appropriate regulators that inhibit CD38 mRNA expression might be a useful approach, especially in $CD38^+$ subset patients. Compared to $CD38^-$ leukemia subset patients, inhibition of CD38 mRNA production in $CD38^+$ leukemia might successfully inhibit cell proliferation and reduce resistance to apoptosis, and hence improve the prognosis.

Collectively, several regulators of CD38 expression have been suggested that mostly regulate CD38 mRNA, such as ATRA, cytokines and hormones. However, regulation of CD38 expression by its substrate, NAD, has not been studied. The novel work in this chapter suggested firstly that CD38 expression might be regulated by manipulating NAD levels, either by inhibiting a key enzyme in NAD biosynthesis, NAMPT, by using FK866 in order to decrease NAD levels or by supplementing cells with NAD to elevate the intracellular levels. Manipulation of NAD levels might be involved in CD38 regulation indirectly through the effect of NAD availability on

sirtuin and PARP, as NAD-consuming enzymes. Alternatively, NAD might be involved in CD38 transcriptional regulation via a protein such as C-terminal binding protein (CtBP). This study has provided the first evidence indicating that controlling NAD levels can attenuate CD38 mRNA expression.

5.2 Materials and methods

5.2.1 Materials

Kuromanin was purchased from Sigma (Poole, UK), NAD was from Melford (Ipswich, UK) and FK866 (APO866, (E)-N-[4-(1-benzoylpiperidin-4-yl) butyl]-3-(pyridin-3yl) acrylamide was from Axon Medchem (Groningen, The Netherlands). Plastic boxes for oxygen exposures and cylinders containing custom mixtures of O_2, CO_2 and N_2 were all obtained from the Diving Diseases Research Centre (DDRC, Plymouth, UK).

5.2.2 Evaluation of CD38 expression in differentiating cells after treatment with kuromanin

Differentiated cells treated with 30 µM kuromanin for 6, 18 and 24 hours were subjected to qPCR analysis for CD38 expression as previously described in Chapter 2, Section 2.7.

5.2.3 Determination of the effects of addition of FK866 or NAD on intracellular NAD levels, cell proliferation and CD38 expression in cell lines

HL60 and RAJI cells (5×10^5 cells ml^{-1}) were incubated in 24-well plates at 37 $^{\circ}$C in RPMI-1640 culture media in the presence or absence of 1-100 µM NAD^+ or 1-1000 nM FK866. After 24 hours cells were removed from each well and used for analysis of intracellular NAD levels and MTT assay (Sections 2.9.2.1 and 2.6 respectively).

CD38 expression in RAJI, HL60 and differentiating HL60 cells were determined in the presence or absence of 100 µM NAD^+ or 100 nM FK866 for 6, 12 and 24 hours incubation at 37 °C, by quantitative real-time qPCR (see Chapter 2, section 2.7). The $\Delta\Delta C_T$ method was used to determine the relative quantity of CD38 mRNA in samples.

5.2.4 Oxygen exposure protocol

HL60 or RAJI cells (1-2 × 10^6 ml^{-1}) were placed in 6-well plates in air tight plastic boxes (21.5 cm × 21.5 cm × 11 cm; total volume five litres) prepared at the DDRC (Fig. 5.1). Boxes were flushed for 5 min with gas mixtures either giving a hypoxic environment (2% O_2, 5% CO_2, 93% N_2 or 5% O_2, 5% CO_2 and 90% N_2) or a hyperoxic environment (95% O_2, 5% CO_2) at a rate of 4 l min^{-1}. The boxes were then sealed and placed at 37 °C in a conventional cell incubator for 30, 60 and 90 min. In each experiment the normoxic controls were incubated under conditions of atmospheric oxygen concentration (21% O_2, 5% CO_2 and 74% N_2). All the cells were grown in RPMI-1640 medium supplemented with 10% FCS.

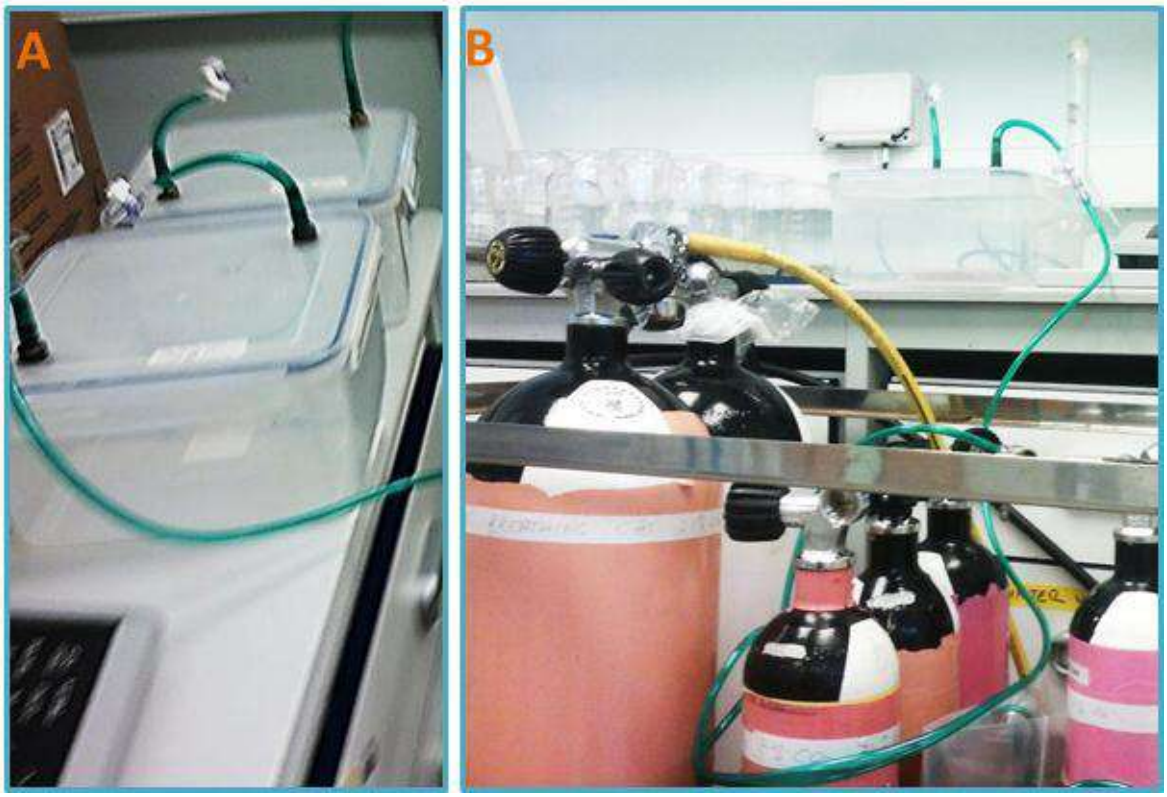


Figure 5.1 (A) boxes and (B) Oxygen cylinders used in normoxia, hypoxia and hyperoxia experiments.

5.2.5 Statistical analysis

Statistical analysis of the data was assessed using Fisher's one way analysis of variance (Statview 5.0.1; Abacus concepts, USA) or Student's t-test as appropriate. Data are expressed as means ± SEM for three separate experiments in triplicate, unless otherwise stated. A difference of $P < 0.05$ was considered statistically significant.

5.3 Results

5.3.1 Effect of elevated NAD^+ levels on CD38 expression after kuromanin treatment

It was found previously (Chapter 3) that kuromanin, the novel human CD38 inhibitor, caused an elevation in intracellular NAD^+ levels. In this Chapter the aim was to establish whether this elevation in intracellular NAD^+ might regulate CD38 mRNA expression. Interestingly, qPCR results revealed that treatment with kuromanin leads to attenuation of CD38 expression.

The results showed significant inhibition of CD38 mRNA expression in differentiating cells with 30 μM kuromanin and started from as early as 6 h differentiation in the presence of kuromanin (Fig. 5.2). A significant drop in mRNA levels was also shown at 18 h and 24 h incubation compared to each control (differentiating cells without kuromanin). In this respect, low CD38 mRNA expression during kuromanin treatment suggested that it might be regulated by intracellular NAD^+.

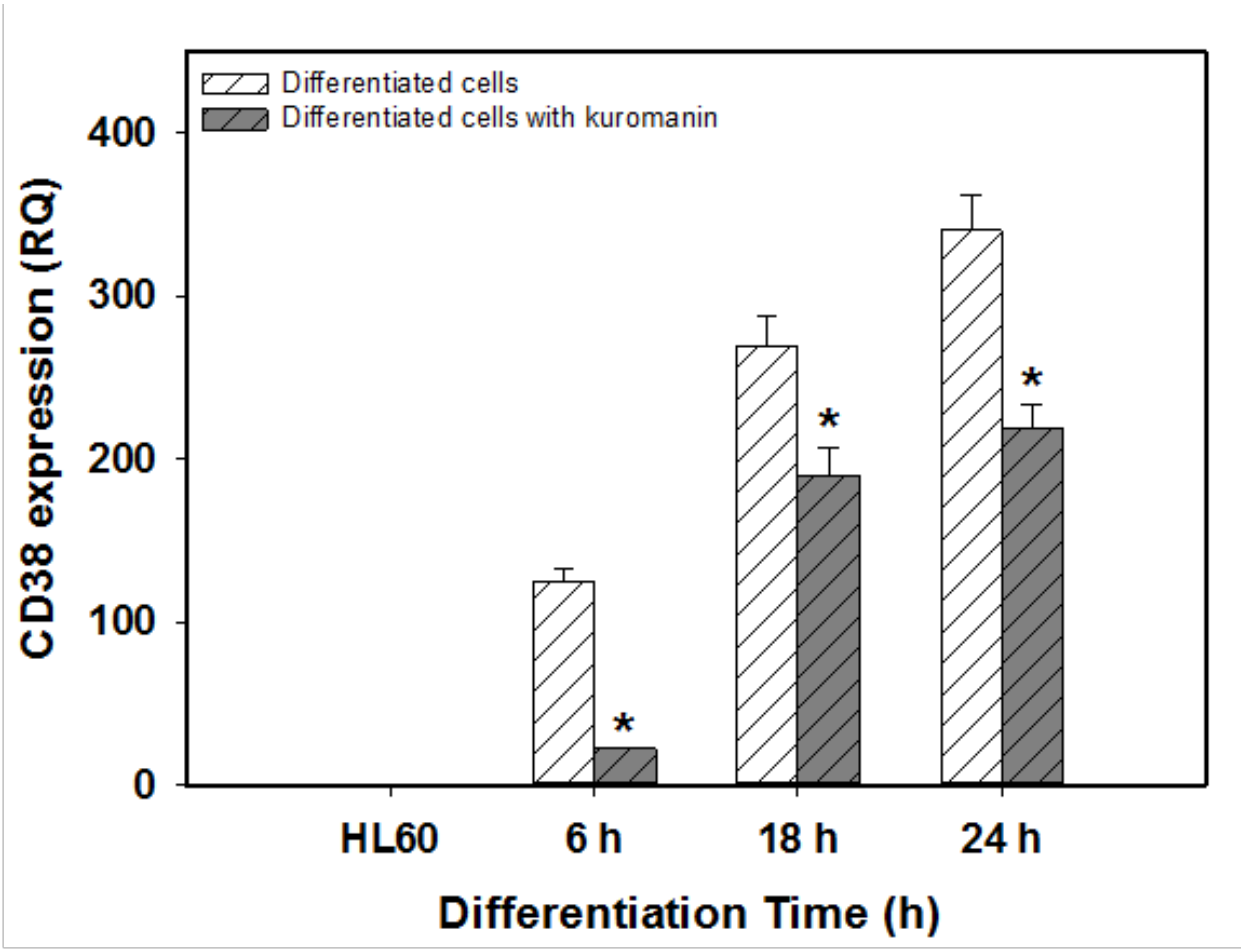


Figure 5.2 Effect of kuromanin (30 µM) on CD38 expression during the time course of differentiation of HL60 cells with 1 µM ATRA up to 24 h comparing to differentiated cells without treatment (as control). Data are means ± SEM, n = 3 (3 measurements per replicate), * denotes a significant difference from each control, $P < 0.05$.

5.3.2 Manipulation of intracellular NAD^+ levels by NAD application or using FK866

To further investigate the kuromanin results, the NAD levels in the cells were manipulated by either elevating NAD^+ levels by NAD^+ application or decreasing the intracellular NAD^+ levels after treatment with FK866, based on previously published reports by Billington *et al.* (2008a; 2008b). In this part of the work, firstly it was tested whether NAD^+ application could be used as a tool to elevate intracellular NAD^+ levels. Hence, HL60 cells and RAJI cells were incubated separately for 24 hours with 0-100 µM extracellular NAD^+ at 37 $^{\circ}$C. The results showed an elevation in intracellular NAD^+ levels that was significant in RAJI cells after 24 h incubation with 30 µM and 100 µM NAD^+, and in HL60 cells with only 100 µM NAD^+ (Fig. 5.3A).

As intracellular NAD^+ levels were elevated, the MTT assay was performed to monitor cell vitality during treatment. The results show that treatment with NAD^+ had an apparent effect on vitality of RAJI cells that was significant with 10 and 100 µM NAD^+ (Fig. 5.3B). In HL60 cells, a similar effect was seen, but it was not statistically significant (Fig. 5.3B). Collectively, an increase in intracellular NAD^+ levels was confirmed both in HL60 and RAJI cells with the extracellular NAD^+ application.

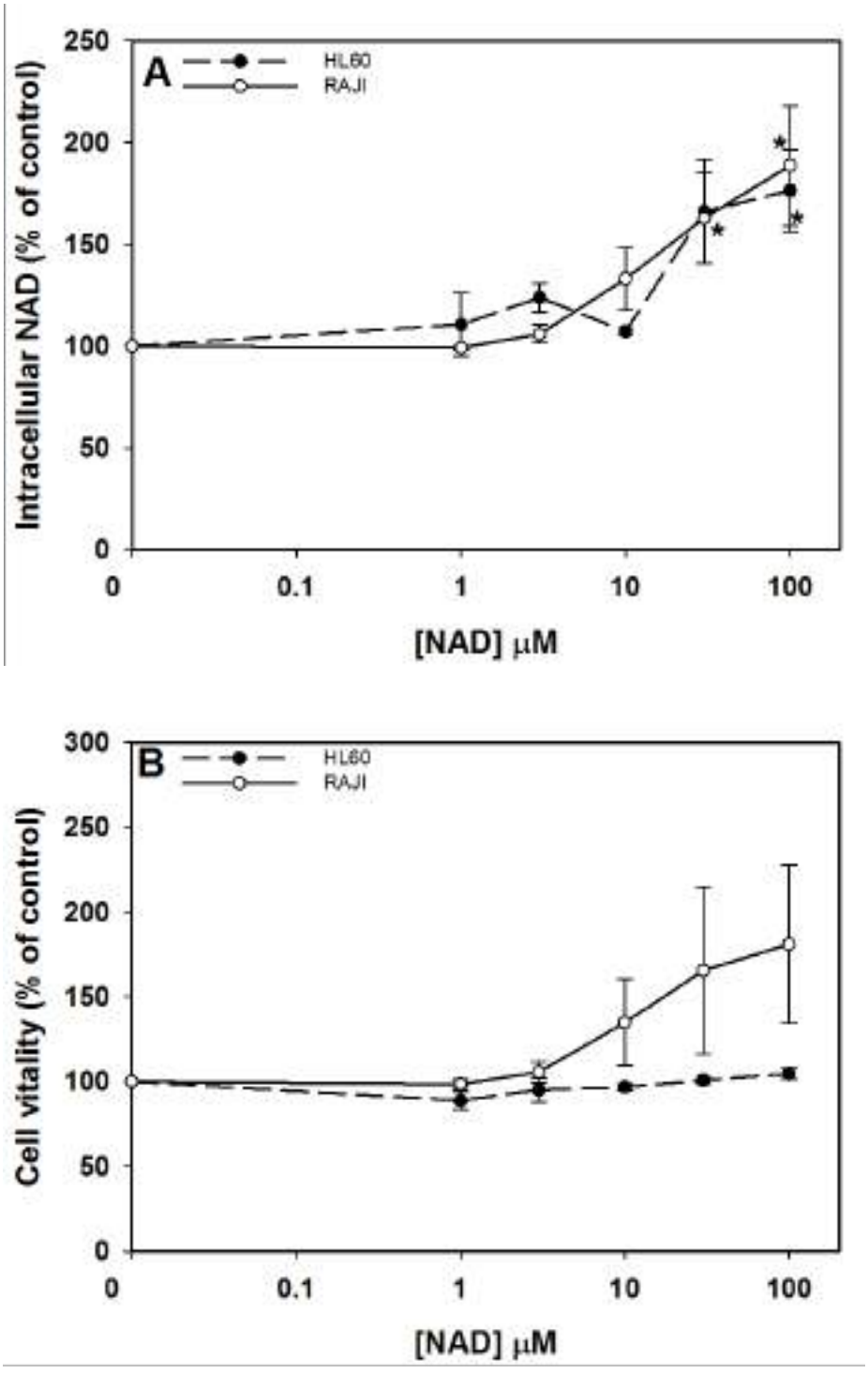


Figure 5.3 Effect of treatment with NAD^+ (0-100 μM) for 1 day on RAJI and HL60 cells comparing to untreated control (100%). (A) Intracellular NAD^+ levels and (B) cell vitality (as determined by MTT assay). Data are means ± SEM, n = 3 (3-4 measurements per replicate). * denotes a significant difference from the control (HL60 or RAJI cells without treatments), $P < 0.05$.

Several FK866 concentrations (0-1000 nM) were also incubated for up to 24 h with HL60 and RAJI cells, and intracellular NAD^+ levels were determined. A significant reduction in intracellular NAD^+ levels in RAJI and HL60 cells was evident after 24 h incubation with all FK866 concentrations (Fig. 5.4 A). Thus, FK866 caused a concentration-dependent decrease in intracellular NAD^+ levels.

Interestingly, intracellular NAD^+ levels in RAJI cells after treatment with FK866 were lower than in HL60 cells; this may be because RAJI cells already showed lower NAD^+ levels compared to HL60 cells accompanied by significantly higher CD38 activity. Furthermore, the MTT results have shown that cell vitality was rapidly depleted after 24 h treatment with FK866 (Fig. 5.4 B). A significant drop in cell vitality was evident both in HL60 and RAJI cells and with 1-1000 nM FK866. Moreover, cell vitality in RAJI cells was lower than that in HL60 cells when assayed using MTT reduction. Altogether, the cell vitality and NAD^+ data in the present study and the effect of treatment with FK866 suggests a high turnover of intracellular NAD^+, and after treatment with extracellular NAD^+ suggests the cell's ability to uptake the extracellular NAD^+.

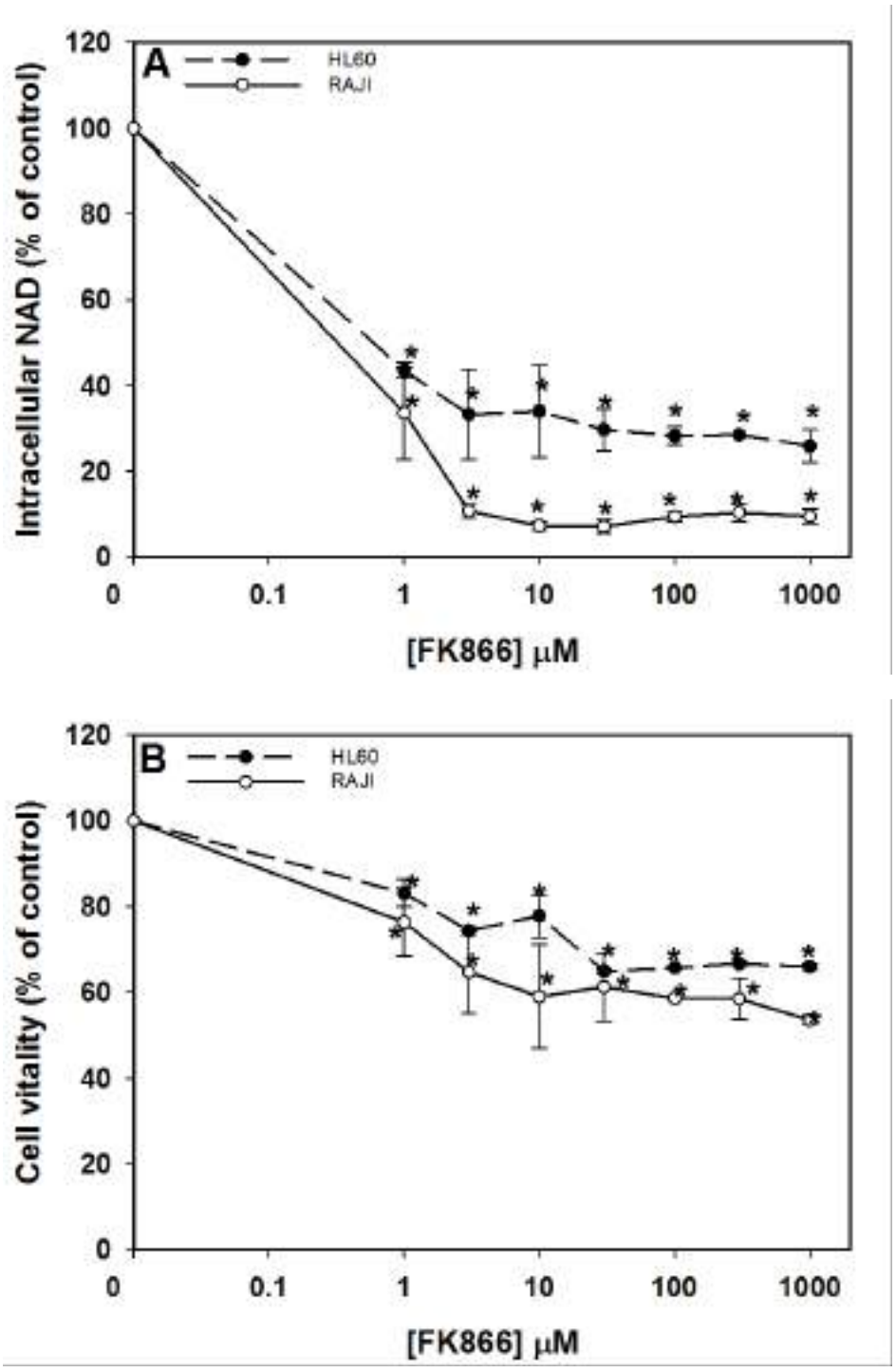


Figure 5.4 Effect of treatment with FK866 for 1 day on RAJI and HL60 cells comparing to untreated control (100%).. (A) Intracellular NAD levels and (B) cell vitality (as determined by MTT assay). Data are means ± SEM, n = 3 (3-4 measurements per replicate). * denotes a significant difference from the control (HL60 or RAJI cells without treatments), $P < 0.05$.

5.3.3 Effect of intracellular NAD^+ levels on CD38 expression

In the above section it was confirmed that intracellular NAD^+ levels in HL60 and RAJI cells were significantly depleted after treatment with FK866 and elevated after extracellular NAD^+ application. It was of interest to test whether decreasing or elevating intracellular NAD^+ levels after treatment with FK866 and NAD^+, respectively, would also participate in the regulation of CD38 expression, in the same way as the effect of intracellular NAD^+ elevation by kuromanin. Therefore, analysis of CD38 gene expression profiles was performed using qPCR in HL60 and RAJI cells (Fig 5.5A and B, respectively), and during the time course of HL60 differentiation (Fig 5.6).

qPCR analysis of HL60 cells (Fig. 5.5 A) demonstrated that the effect of NAD^+ (100 µM) or FK866 (100 nM) was not the same at each time point, since an apparent decrease in CD38 expression at 24 h ($P > 0.05$) was found with all treatments, compared to the control (untreated HL60 cells). Also, there was a moderate, but not significant, increase in CD38 expression at 6 h and 12 h incubation with FK866 and NAD^+. However, FK866 or NAD^+ application had a similar effect on CD38 expression in HL60 cells.

This experiment was also performed with RAJI cells with comparable results to those with HL60 cells (Fig. 5.5 B). A visible attenuation in CD38 expression was shown at 12 h ($P > 0.05$) and at 24 h ($P < 0.05$) after treatment with FK866 and NAD^+. However, at 6 h, a notable rise in CD38 expression after treatment with NAD^+ was observed (but not with FK866) compared to the control. Overall, an apparent decline in CD38 mRNA expression was shown in both HL60 and RAJI cells, particularly after 24 h incubation with FK866 or NAD^+ application.

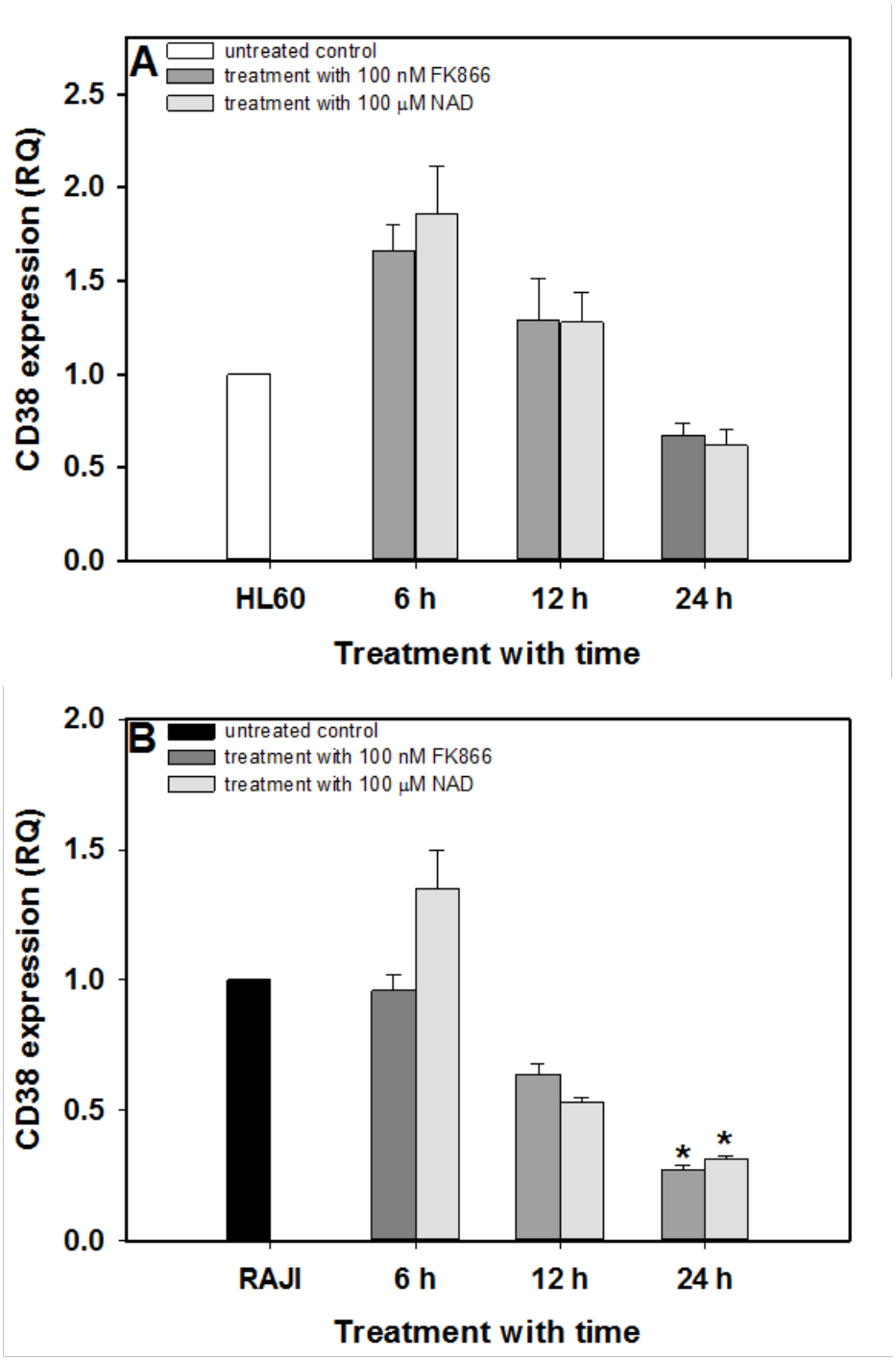


Figure 5.5 Effect of treatment with 100 nM FK866 and 100 μM NAD^+ on CD38 expression after 1 day incubation comparing to the control (untreated HL60 and RAJI cells) in (A) HL60 cells, and (B) RAJI cells. Data are means ± SEM, n = 3 (3 measurements per replicate). * denotes a significant difference from the control (RAJI cells without treatments), $P < 0.05$.

To further investigate the effect of manipulating NAD^+ levels on the transcriptional regulation of CD38 expression, a similar experiment was also performed in differentiating HL60 cells by incubating the cells with FK866 or NAD^+ up to 24 h. The data demonstrated a significant attenuation in CD38 expression at 6, 12 and 24 h of treatment with FK866 or NAD^+ compared to untreated controls (differentiating cells, Fig. 5.6). A similar effect was observed with both treatments. Overall, attenuation in CD38 expression was clearly shown in cells treated with FK866. However, unexpectedly, the results showed that CD38 expression dropped rapidly even with increasing intracellular NAD^+. The results of NAD^+ application are consistent with the kuromanin results, showing an inhibition of CD38 expression when intracellular NAD^+ levels were elevated.

Hence, whether NAD^+ is elevated or increased, attenuation in CD38 expression may have been occurring. This strongly confirmed a possible role of NAD^+ levels in the regulation of CD38 expression. These observations suggested that NAD^+ metabolism might be considered as a novel target for regulating CD38 expression.

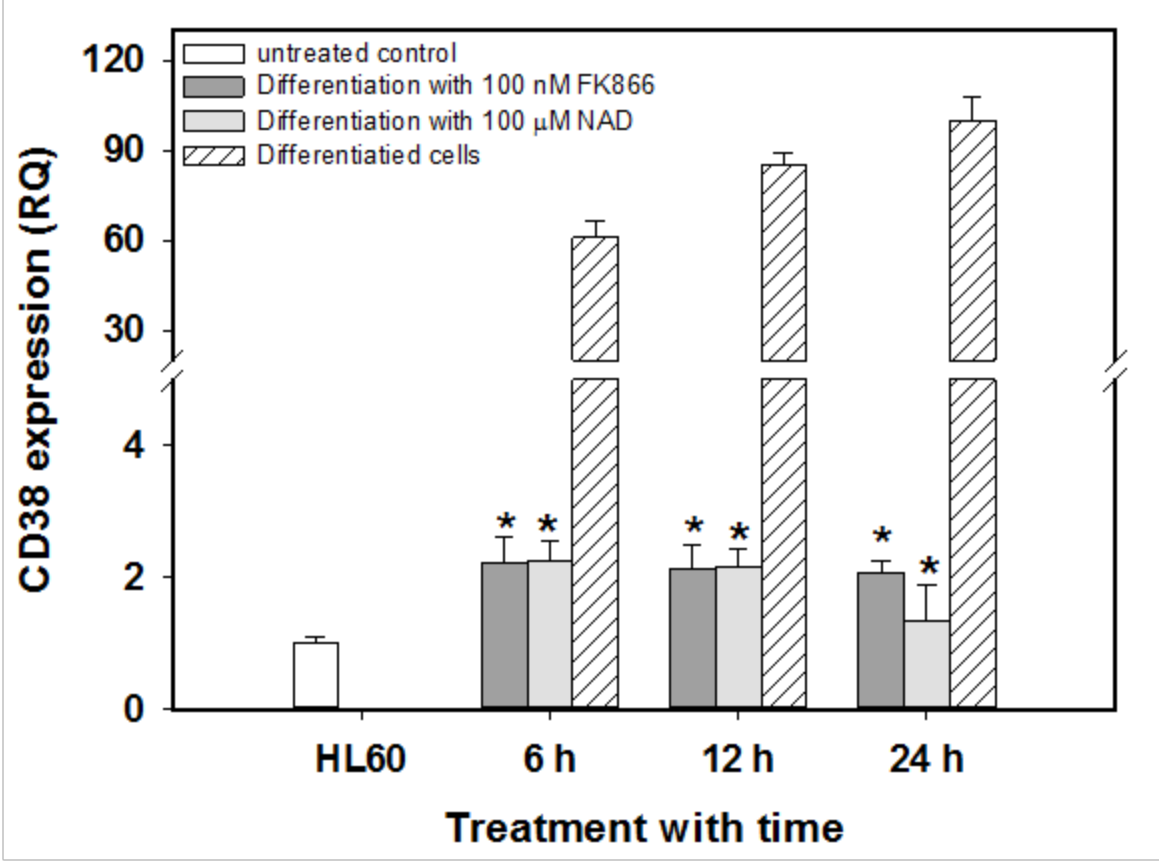


Figure 5.6 Effect of treatment with 100 nM FK866 and 100 µM NAD^+ on CD38 expression after 1 day incubation in differentiating cells (ATRA treated cells) up to 24 h comparing to HL60 and ATRA treated cells without FK866 or NAD. Data are means ± SEM, n = 3 (3 measurements per replicate). * denotes a significant difference from the appropriate control (differentiated cells without treatments), $P < 0.05$.

5.3.4 Effects of hypoxia and hyperoxia conditions on CD38 expression in leukaemia cell lines

Hypoxia, a decrease in oxygen levels, is a hallmark of human cancer cells *in vivo* (Harris, 2002). For instance, leukaemia cells in bone marrow are considered physiologically hypoxic (Harrison *et al.,* 2002). In the current study, the malignant cells, HL60 and RAJI cells, which are derived from human leukaemia cells and lymphoma cells respectively, might also be adapted to proliferate in a low-O_2 environment. However, these cells were cultured *in vitro* under 21% O_2 (normoxia). This study aimed to culture these cells under conditions that mimic the *in vivo* environment in order to determine the effect of this environment on CD38 expression. As shown in Figure 5.7, RAJI cells were cultured under hypoxia (2% O_2) and incubated for 30 and 90 min and CD38 expression was determined compared to normoxia conditions (21% O_2). CD38 expression, as measured by qPCR analysis, was slightly increased under hypoxia 2% O_2 at 30 min and 90 min incubation times compared with normoxia, but this was not significant ($P > 0.05$). It is important to note that the level of O_2 (2% hypoxia) used was based on previous reports indicating that most cells can be maintained when cultured under these conditions (Han *et al.,* 2006). These data might suggest a link between hypoxia and CD38 expression in the leukaemia cell line. However, the vitality results (Fig. 5.9B) showed no changes in cell vitality as assessed by MTT assay at 30 min under 2% O_2 hypoxia in RAJI cells compared to normoxia.

CD38 expression was also determined in HL60 cells under 2% O_2 and 5% O_2 (simulating hypoxia), and for 30 min, 90 min and 6 h incubation times. Interestingly, a significant effect at 2% O_2 compared to 5% O_2 was observed on CD38 expression (Fig. 5.8). Furthermore, the data showed a strong and significant effect of 2% O_2 hypoxia at 30 min on regulation of CD38 expression in HL60 cells compared to 90 min and 6 h with both 2% O_2 or 5% O_2 ($P < 0.05$). An

apparent decrease in cell vitality was detected under hypoxia (2% O_2) at 30 min incubation time ($P < 0.05$; Fig. 5.9 B).

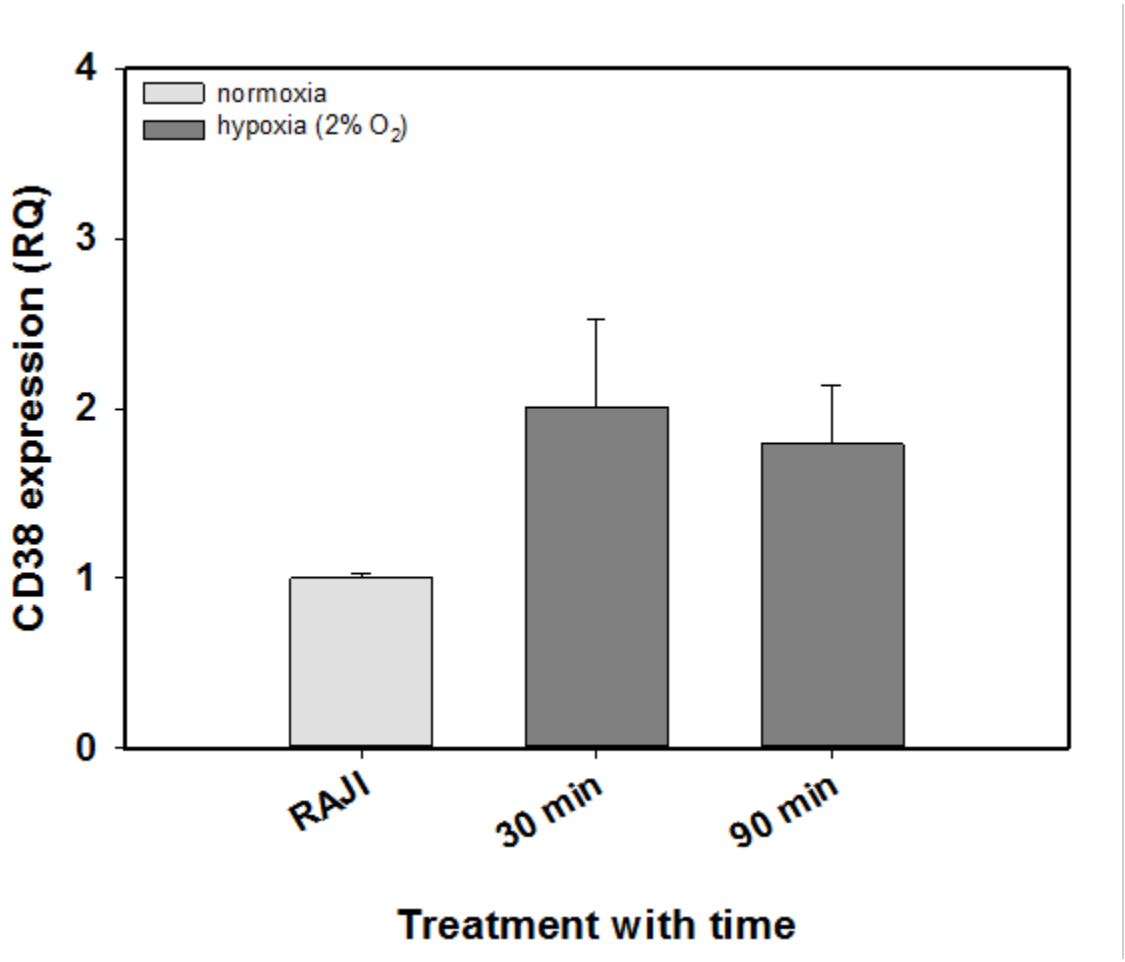


Figure 5.7 CD38 expression in RAJI cells exposed to hypoxia (2% O_2) and incubated for 30 min and 90 min compared to the untreated control (normoxia). Data are means ± SEM, n = 3 (3 measurements per replicate), no significant differences between groups were found ($P > 0.05$).

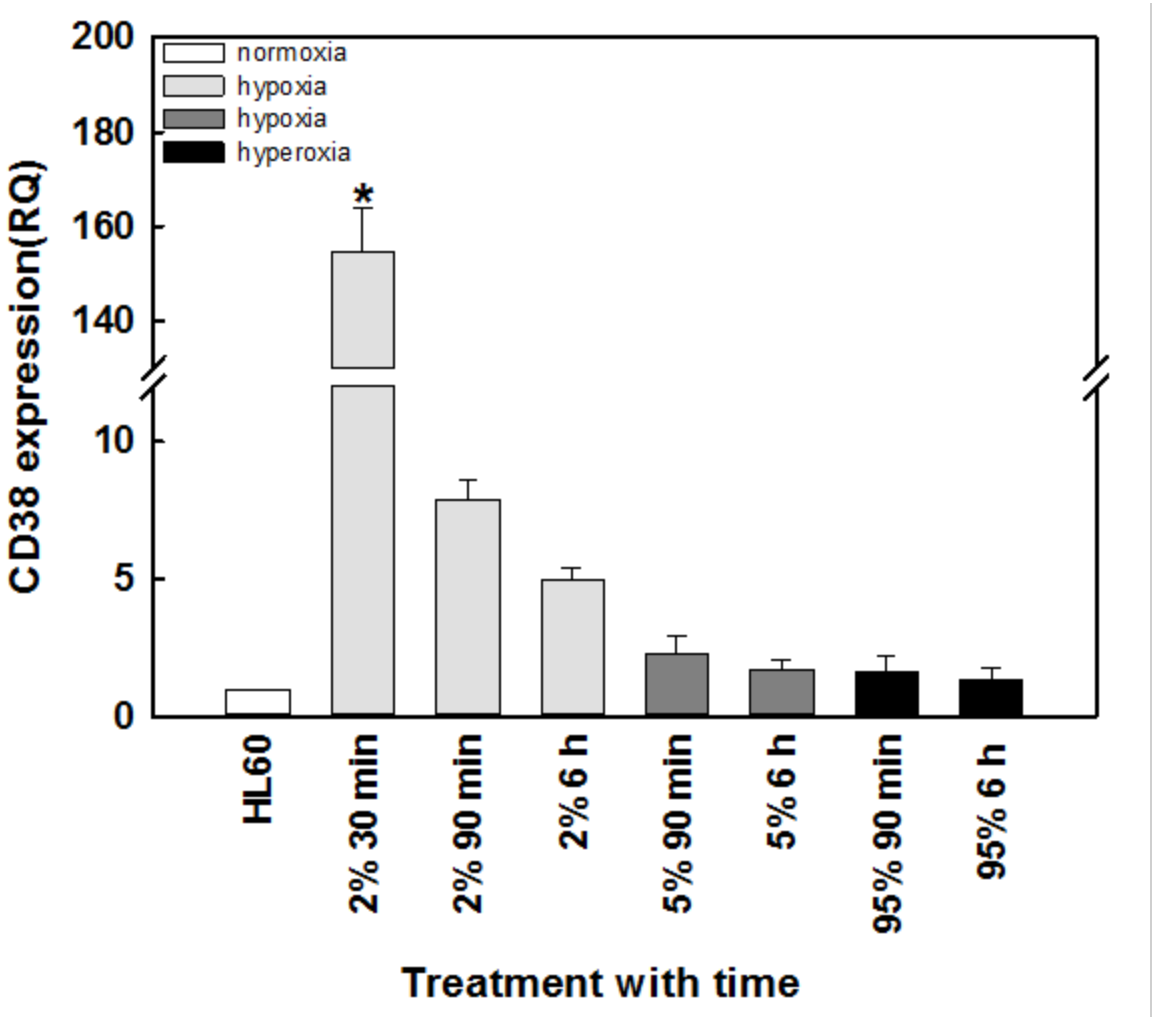
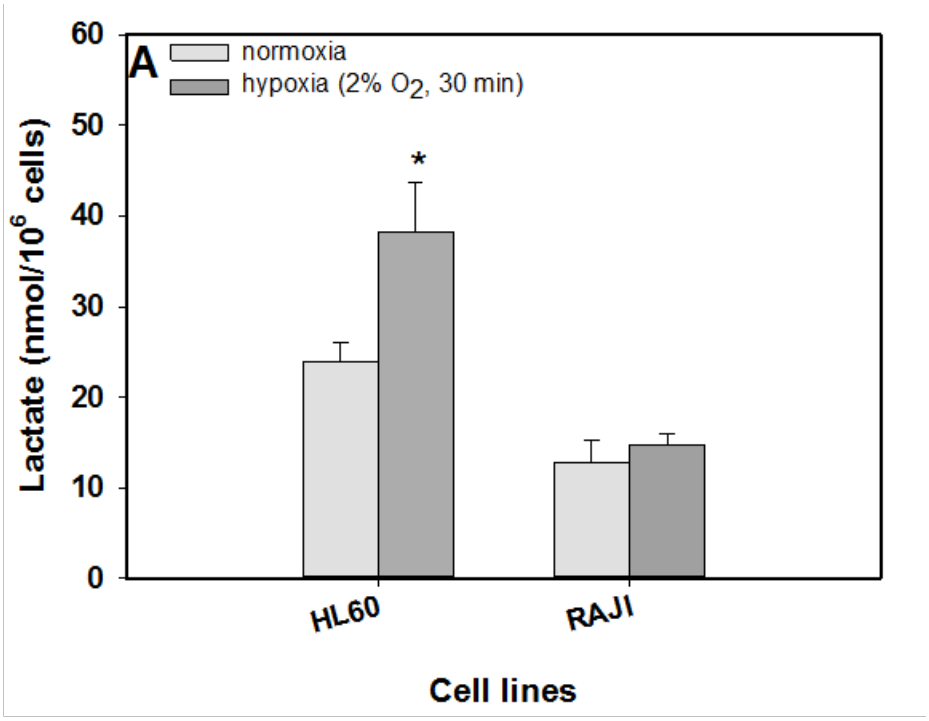


Figure 5.8 CD38 expression in HL60 cells exposed to hypoxia (2% O_2) and incubated for 30 min, 90 min and 6 h compared to the control (normoxia), hypoxia (5% O_2), and hyperoxia (95% O_2). Data are means ± SEM, n = 3 (3 measurements per replicate). * denotes a significant difference from the control (HL60 cells without treatments), $P < 0.05$.

It is thought that the cell vitality assay measures mitochondrial dehydrogenase activity (Mosmann, 1983). However, under hypoxia the metabolism of normal or cancerous cells shifts from the aerobic pathway to lactic fermentation (Warburg, 1956) rather than oxidative phosphorylation. Therefore, one might expect that dehydrogenase activity may be affected under these conditions, so that under hypoxia (2% O_2), the measured cell vitality is decreased. In addition to investigating CD38 expression under hypoxia, it was also investigated under 95% O_2 (hyperoxia) at 90 min and 6 h incubation (Fig. 5.8). As expected, there was no effect of hyperoxia on CD38 expression. Collectively, these novel data strongly confirmed the effect of low oxygen tension on CD38 expression in leukaemia cells.

The effect of hypoxia on glycolysis activity was evaluated by measuring lactate production in both leukaemia cell lines. Figure 5.9 A clearly illustrates that under hypoxia (2% O_2, 30 min incubation), HL60 cells significantly increased lactate levels compared to RAJI cells or to normoxia. These data might suggest a link between hypoxia, CD38 expression and glycolysis activity.



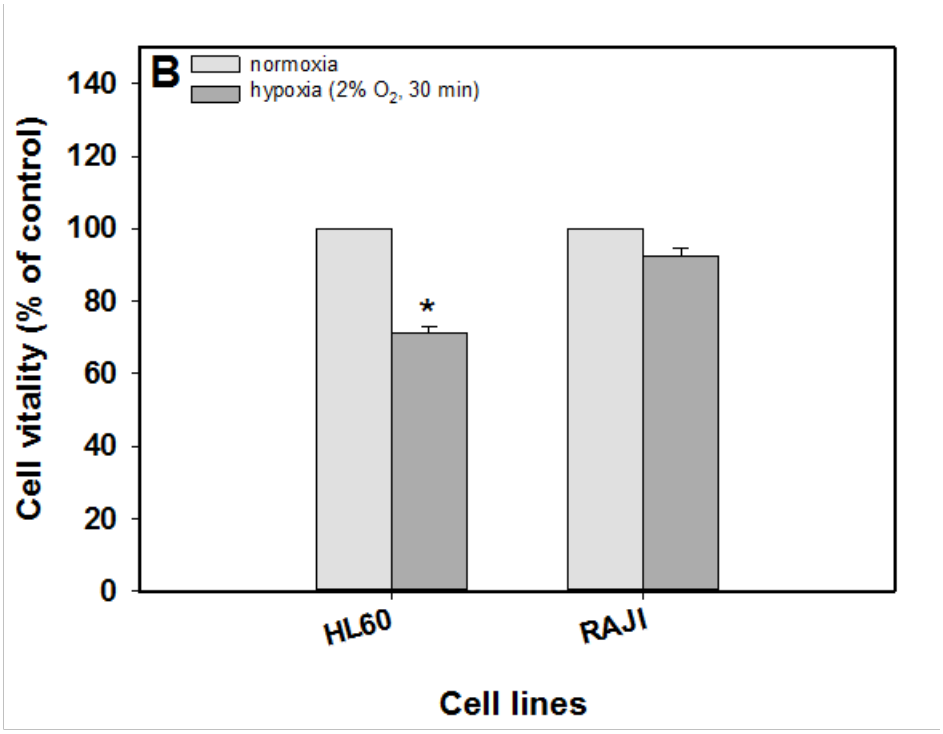


Figure 5.9 Effect of hypoxia (2% O_2) after 30 min incubation in both HL60 and RAJI cells on (A) lactate production, n = 3 (2 measurements per replicate), and (B) cell vitality (MTT assay) comparing to each untreated control (normoxia), n=2 (3 measurements per replicate). Data are means ± SEM.* denotes a significant difference from the related control (HL60 or RAJI cells without treatment), $P < 0.05$.

To further characterize the hypoxic response in the cells, it seemed important to evaluate intracellular NAD^+ levels in addition to evaluating the effects of low O_2 tension on cell vitality and lactate levels *in vitro*. Therefore, and also to determine whether NAD^+ levels might be playing a role in the upregulation of CD38 under the hypoxic conditions, the same experiment was repeated and NAD^+ levels were assayed by the routine NAD cycling assay.

The results show that lower NAD^+ levels were observed under hypoxic conditions (2% O_2) at 30 min incubation time in HL60 cells compared to the normoxia, while RAJI cells did not show any change in NAD^+ levels (Fig 5.10). The drop in NAD^+ levels (in HL60) might be mediated by CD38 upregulation as confirmed under the same conditions in HL60 cells. However, CD38 activity might not be regulated at 30 min under hypoxia; there might be other mechanisms responsible for the decline in NAD^+ levels, such as a high glycolysis activity. Interestingly, the decrease in NAD^+ levels was concomitant with the decrease in the cell vitality (MTT) in HL60 cells and under hypoxic conditions (Fig. 5.9 B).

Altogether, the results indicate that the hypoxic response of human leukaemia cells is characterized by a rapid but transient increase in CD38 expression and lactate production, but with a significant decrease in intracellular NAD^+ levels and not with a significant decrease in cell vitality. Interestingly, the expression of CD38 in $CD38^-$ cells (HL60) under hypoxia (2% O_2) was greater than that of $CD38^+$ cells (RAJI) at 30 min incubation time.

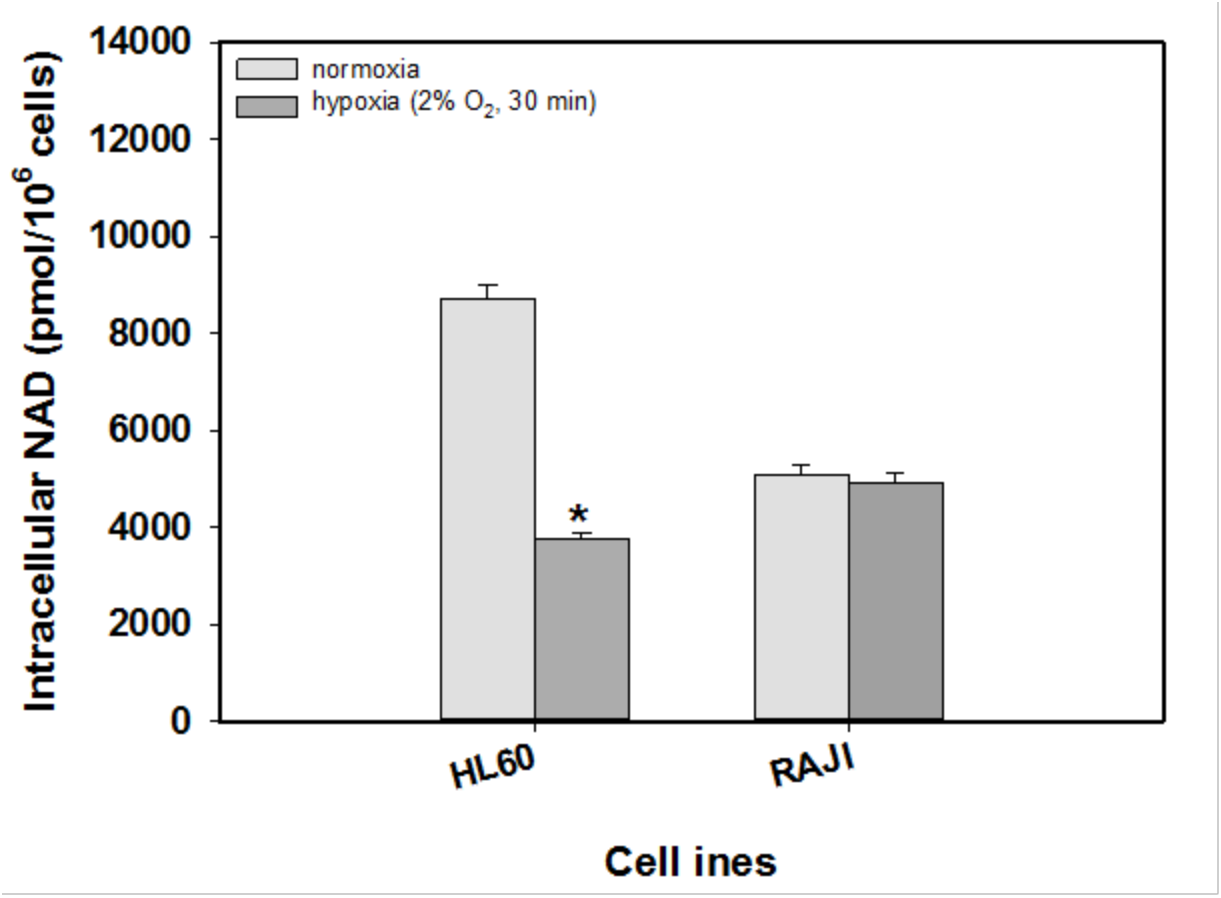


Figure 5.10 Effect of hypoxia (2% O_2) after 30 min incubation both in HL60 and RAJI cells on intracellular NAD^+ levels comparable to the untreated control (normoxia). Data are means ± SEM, n = 3 (2 measurements per replicate). * denotes a significant difference from the control (HL60 cells without treatments), $P < 0.05$.

5.4 Discussion

Several mechanisms of regulation of CD38 expression that involve transcriptional and post-transcriptional levels of gene expression have been previously reported. Interestingly, the analysis of the human CD38 gene has revealed the presence of a number of response elements, for example, the retinoic acid response element (RARE; Tirumurugaan *et al.,* 2008). These make this gene responsive to a variety of physiological stimuli, suggesting the complex nature of CD38 expression in various types of mammalian cells. For instance, a comparatively rapid increase in CD38 expression occurs in response to ATRA in HL60 cells, and the mechanism that mediates this regulation has been previously studied (Munshi *et al.,* 2002). Transcriptional regulation of CD38 expression was also studied during ATRA-induced HL60 differentiation in the work described in this Chapter, but after incubation with kuromanin. It was seen that treating the differentiating cells with kuromanin inhibits CD38 mRNA expression compared to the untreated control. There are two suggested mechanisms that might explain this attenuation in CD38 expression. Firstly, kuromanin inhibition of CD38 cyclase activity might have consequences on the inhibition of CD38 expression. Secondly, the elevation in intracellular NAD^+ following inhibition of CD38 cyclase activity might have an impact on CD38 expression, since NAD^+ may affect gene expression through two pathways: (1) through the NAD^+-consuming enzymes, PARP-1 and sirtuins, that can affect several transcriptional factors (D'Amours *et al.,* 1999; Ford *et al.,* 2006) and (2) through the alterations in NAD levels that might modulate an important NAD(H)-dependent transcription co-repressor, the C-terminal binding protein (CtBP). The change in NAD levels may regulate the dehydrogenase activity of CtBP as well as affect the binding of CtBP to specific repressor complexes (Kumar *et al.,* 2002). It is important to note that repressors require an association with corepressors to mediate

inhibition of gene transcription (Tyler and Kadonaga, 1999). Hence, the elevation of NAD^+ levels might mediate CD38 mRNA inhibition through regulation of CtBP.

In addition to the kuromanin results, it was investigated whether CD38 expression is regulated by NAD^+ levels by using NAD^+ application and FK866. It was hypothesized that the elevation in intracellular NAD^+ levels following NAD^+ application might induce CD38 expression, in order to degrade the high NAD^+ levels and vice versa with FK866. However, the results unexpectedly showed that elevation of intracellular NAD^+ by extracellular NAD^+ application or inhibition intracellular NAD^+ in all cell lines resulted in attenuation of CD38 expression. One explanation is that CD38 might be regulated by specific concentrations of NAD^+, but not by 100 µM extracellular NAD^+ or by using 100 nM FK866 to diminish NAD^+ levels. These concentrations might be completely different from the normal levels in cells, which are suggested to be around the high micromolar range (Yang *et al.,* 2007). Therefore, investigations of CD38 expression in cells incubated with a range of concentrations of NAD^+ are suggested for further studies. It is worth mentioning that initial studies found that treatment of the $CD38^+$ cells with NAD^+ was shown to induce inactivation of CD38 activities; cyclase, hydrolase (Han *et al.,* 2000) or CD38 might undergo extensive self-oligomerization in the presence of NAD^+ (Guida *et al.,* 1995). Hence, NAD^+ application inhibited CD38 activities in previous studies, but the effect of NAD^+ application on CD38 mRNA was not investigated. Furthermore, CD38 expression was also investigated, in this study, after inhibition of intracellular NAD^+. NAD^+ levels were inhibited by FK866 through its impact on the NAD biosynthesis enzyme (NAMPT). Interestingly, low intracellular NAD^+ was accompanied by an inhibition of CD38 expression. This downregulation of CD38 expression might also be NAD-dependent. One of the possible explanations is that limited availability of the substrate NAD for the CD38 enzymatic activities might result in

reduction in both NAD-hydrolase and cyclase activities, which further leads to the control of CD38 expression. It is important to note that FK866 causes depletion of NAD^+ levels which results in cell death by autophagy as confirmed by Billington *et al.* (2008b). Thus, cells incubated for 24 h with this agent, to ensure that NAD^+ levels dropped first, and to keep the cells viable in order to investigate the effect of inhibiting the NAD recycling pathway on CD38 expression.

Previous results (Chapter 3) suggested that NAD levels are CD38-dependent. Intriguingly, this study suggests that CD38 expression might also be NAD-dependent. The current findings have provided the first *in vitro* evidence that NAD^+ metabolism might be a novel target for controlling CD38 expression by using FK866 and extracellular NAD^+ application strategies in addition to using kuromanin. Targeting NAD metabolism by using NAD^+ application might serve as a treatment strategy in cancer, Huntington's disease, multiple sclerosis, and neurodegenerative diseases (Khan *et al.*, 2007). Targeting NAD metabolism by using FK866 might also implicated in cancer therapy (Hasmann and Schemainda, 2003; Holen *et al.,* 2008). Indeed, regulation of CD38 expression via manipulation of NAD levels might serve as a treatment strategy for leukemia patients, since CD38 works as a dependable marker of unfavourable prognosis and as an indicator of cell proliferation and activation (Malavasi *et al.*, 2011). Moreover, inhibition of CD38 expression might reduce the consequences of the CD38-CD31 interaction, and supramolecular complex signalling that mediated CLL homing processes and survival. Hence, controlling CD38 expression in leukemia patients might affect the unfavourable prognosis and consequently inflict on patient survival.

Regulation of CD38 expression in leukaemia cells was further investigated under hypoxia (similar to *in vivo* conditions). Hypoxia, a decrease in oxygen levels, plays a major role in many pathological processes such as ischemic stroke and tumour progression (Harris, 2002). Under hypoxia, cells may survive and adapt to the hypoxic environment. These adaptive responses of cells to hypoxia may involve the induction of specific gene expression which may help to suppress or limit the effects of hypoxia on these cells (Yun *et al.,* 1997). Several studies have showed a significant association between hypoxia and CD38 activity in disease states, in several types of cells. For instance, a change in CD38 activity that is associated with hypoxic pulmonary vasoconstriction (HPV) has been indicated (Wilson *et al.,* 2001). It was suggested that hypoxia-mediated vasoconstriction is cADPR dependent and the mechanism was attributed to increase an $NADH:NAD^+$ ratio (due to increased NADH formation via glycolysis) that appears to favour the net production of cADPR probably because of the inhibition of cADPR hydrolase activity of CD38 (Wilson *et al.,* 2001; Kotlikoff *et al.,* 2004). cADPR accumulation might also suggest activation of ADP-ribosyl cyclase activity. CD38 expression was also found to be changed under hypoxia in brain cells from rats (Salmina *et al.,* 2008). It is important to mention that regulation of CD38 activities might reflect regulation of its mRNA expression. In addition to that, hypoxia attenuated CD38 expression in pancreatic β-cells from HIT-T15 hamsters (Ota *et al.,* 2012). Recently, an association between CD38 and activation of hypoxia inducible factor (HIF), a family of transcription factors extensively involved in the response of mammalian cells to low oxygen tension, was shown in allergic airway disease (So Ri *et al.,* 2011). However, regulation of CD38 expression under hypoxia and in leukaemia cells is still poorly studied. The current novel results successfully demonstrate that hypoxia induces the expression of CD38 in leukaemia cells. The data has shown that different leukaemia cells exhibit different levels of sensitivity to

hypoxia (2% O_2); there was more regulation of CD38 mRNA in HL60 cells in response to hypoxia compared to RAJI cells, although CD38 expression in untreated RAJI cells was higher than in HL60 cells. Indeed, leukaemia cells in bone marrow are considered physiologically hypoxic, with oxygen levels approximately three times lower than that usually applied during *in vitro* cell culture (Harrison *et al.*, 2002). Therefore, the hypoxic condition in a current study was within the range for these environments. The reason for using hypoxic conditions was that under hypoxia a drop in NAD levels might occur. Hence, and in line with the FK866 results, it was hypothesized that the decline in NAD levels (as a consequence of lactic fermentation) might also mediate CD38 mRNA downregulation. However, the results unexpectedly showed upregulation of CD38 mRNA expression. This might raise the possibility that NAD levels might also be involved in this interesting regulation. Another possibility is that tumour cells may require increased expression of CD38 to maintain cell survival and resistance to apoptosis under hypoxic conditions, and targeting this molecule may be useful for cancer prevention and treatments. Other suggested mechanisms might be associated with hypoxia-induced cytokines. For instance, the release of TNF-α, expected to have occurred under hypoxia as previously shown in retinal ganglion cells (Hong *et al.*, 2008), might be linked to CD38 overexpresion, since TNF-α has been shown to induce CD38 expression (Barata *et al.*, 2004). However, further studies are needed to clarify the mechanism of the hypoxia induced upregulation of CD38 in leukaemia cells. The consequences of a hypoxic environment on cell physiology, that is reflected by more lactate production (from lactic fermentation) with lowered intracellular NAD levels, might cause metabolic dysfunction. Thus, the possible reason that CD38 protein is frequently overexpressed in a leukemia might be because leukaemia cells, *in vivo*, exist under constant hypoxic conditions

and that this might be participating in the development of poor prognosis and metabolic dysfunction in leukaemia cells.

It is worth noting that under hypoxia (2% O_2) for 30 min, CD38 expression levels were greater than under all other conditions. Hence, the glycolysis state was investigated by measuring both lactate and NAD^+ levels, and cell vitality only under these particular conditions. Furthermore, lactate levels were measured as an indicator of glycolytic activity under hypoxia, thus, there was no need to reconfirm the hypoxic conditions by measuring HIF, as most studies do. Ultimately, although the mechanism of hypoxia-induced CD38 regulation was not investigated, but it seems that NAD levels might be involved in this process (Fig. 5.11).

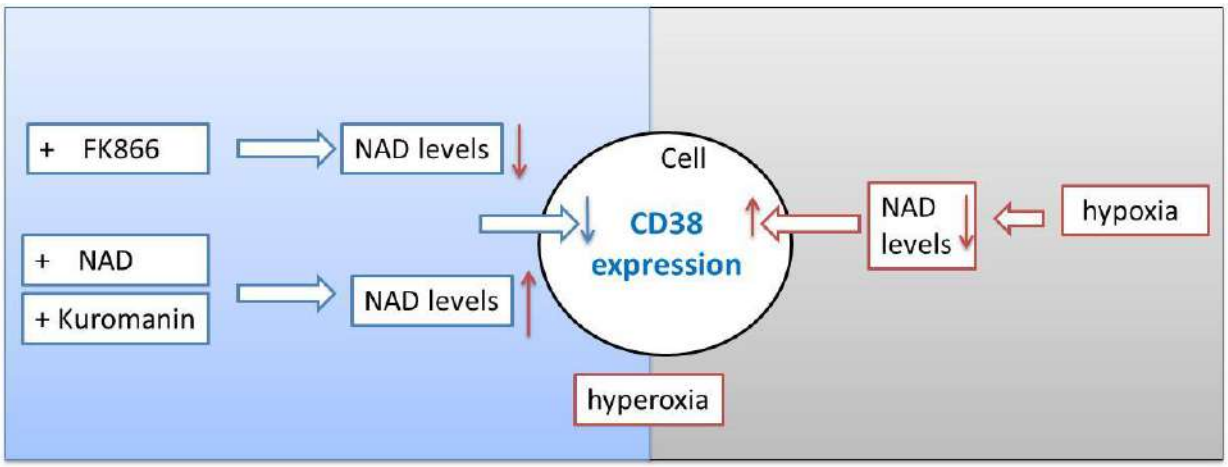


Figure 5.11 Schematic diagram showing how NAD levels might regulate CD38 expression through multiple suggested mechanisms. For instance, elevated NAD levels following kuromanin and NAD^+ application might inhibit CD38 mRNA expression. Alternatively, depletion of NAD by using FK866 might also inhibit CD38 mRNA expression, while decreased NAD levels under hypoxia might upregulate CD38 expression.

Interestingly, under 95% hyperoxia the results did not show any effect on CD38 regulation unlike those seen with hypoxia in leukaemia cells, since it reversed the action of hypoxia on CD38 expression. One of the possible explanations of these results is that the leukaemia cell lines are adapted to function in hypoxic environments, rather than under hyperoxic conditions, and that therefore a hyperoxic environment might not regulate CD38 expression. Indeed, limited studies have investigated the effect of hyperoxia in HL60 cells; an earlier report confirmed that hyperbaric oxygen induces spontaneous and chemotherapy-induced apoptosis in Jurkat and HL60 cells (Ganguly *et al.*, 2002). Other studies have documented the use of hyperoxia as a potential anticancer therapeutic (Henk *et al.*, 1977; Watson *et al.*, 1978), while there are no studies of the effect of hyperoxia on CD38 expression in human cell lines.

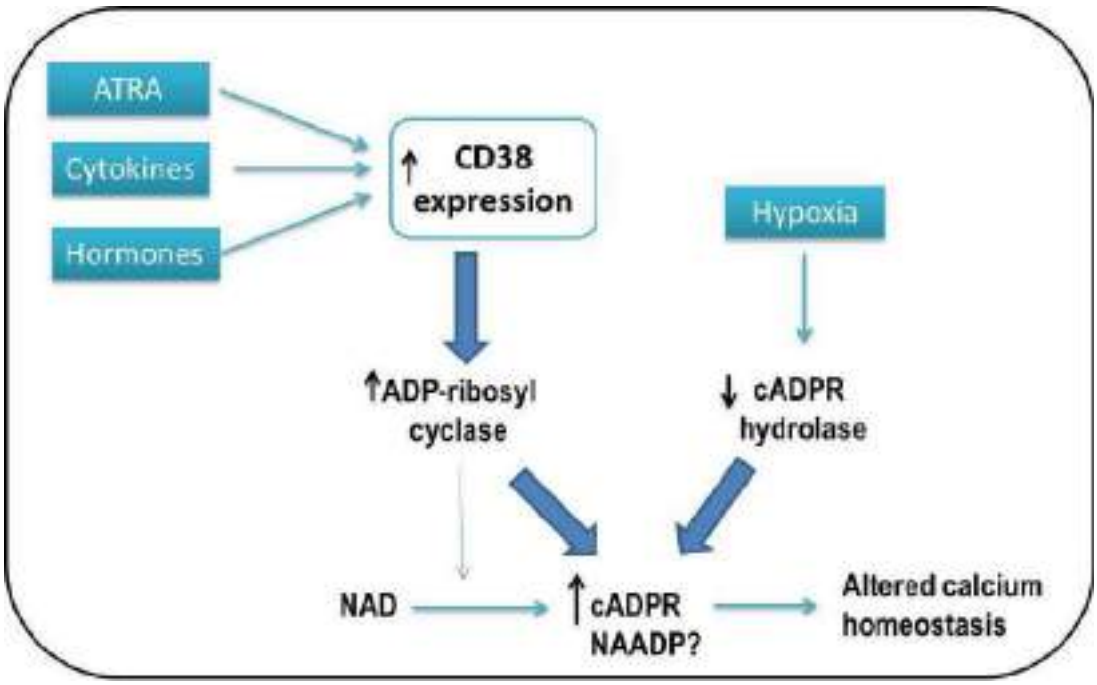


Figure 5.12 CD38 regulations in different cell types by hormones, cytokines, and retinoic acid and the associated increase in ADP ribosyl cyclase activity. In addition to decreasing cADPR hydrolase activity under hypoxia and the consequences of cADPR accumulation, adapted from Kotlikoff *et al.* (2004).

Collectively, regulation of CD38 expression under different stimuli, such as hormones, cytokines, ATRA, hypoxia (Fig. 5.12) or by manipulating NAD levels (Fig. 5.11) are key points for investigation. This might lead to regulation of CD38 activities, or its products, cADPR and NAADP, and their related functions, since the role of CD38 or CD38/cADPR signalling in regulating different cellular functions in humans and animal models has been well investigated. For instance, cADPR plays an important role in hypoxic pulmonary vasoconstriction; HPV (Dipp and Evans, 2001). In diabetes, CD38 plays a distinct regulatory role in the murine model with regards to insulin secretion via calcium mobilization of cADPR-sensitive stores (Kato *et al.*, 1995). The crucial role of CD38 deficiency on prevention of the development of obesity through activation of SIRT/PGC1α has also been documented (Baur *et al*., 2006). Finally, in CLL high levels of CD38 expression correlate with both disease stage and poor prognosis (Morabito *et al.*, 2002). Studies have also confirmed that CD38 is a master regulator of CLL cell homing (Vaisitti *et al.*, 2011). Indeed, therapeutic applications that target CD38 have been more explored in leukemia.

Thus, the current study might prove useful to future researchers especially when they are investigating mechanisms that regulate CD38 expression as a target in leukemia therapy or in other CD38-related diseases.

CHAPTER 6

EFFECT OF LOW NAD LEVELS ON THE DNA DAMAGE AND CELL DEATH

6.1 Introduction

In addition to the well-known functions of NAD in metabolism or as a substrate for CD38, it also participates in DNA repair and cell death via PARP, a family of enzymes with 17 members of which PARP-1 is the founding member (Ying *et al.,* 2005; Hassa and Hottiger 2008). These enzymes catalyze the covalent attachment of poly ADP-ribose (PAR) polymers either to themselves or to other acceptor proteins, using NAD^+ as a donor of ADP-ribose units, in addition to the release of nicotinamide (Hassa and Hottiger 2008). The poly ADP-ribosylation of specific target proteins is crucial for genome stability, DNA repair, telomere maintenance and cell death (Khan *et al.,* 2007). PARP-1 activation is critical in determining cellular fate after DNA damage has occurred (Pieper *et al.* 1999) since, through its role in DNA repair, PARP-1 activation may serve to rescue damaged cells, preventing them from death (Chatterjee *et al.,* 1999). However, extensive DNA damage results in PARP hyperactivation, leading to a rapid depletion of cellular NAD^+ and lowered ATP production, ultimately leading to cell death (Alano *et al.,* 2004; 2010). Cell death by autophagy has also observed when intracellular NAD levels are decreased by using FK866, a NAD-depleting drug (Billington *et al.,* 2008b).

Thus, researchers have shown an increased interest in the area of influence of NAD^+ status on genomic stability, DNA repair and apoptotic cell death, specifically in cancer (Schwartz *et al.,* 1974). The reason for this interest is that cancer cells, which mostly depend on lactic fermentation for ATP production, exhibit a particularly high sensitivity to DNA damage and PARP-1 activation (Zong *et al.,* 2004). For this, an adequate level of cellular NAD in cancer cells is necessary because of a high rate of NAD^+ turnover due to elevated ADP-ribosylation activity (Burkle, 2005). Several studies have drawn attention to the effects of decreases or

increases in NAD levels on PARP activity and cell death (Ying *et al.,* 2003; Benavente *et al.,* 2012). Other cancer therapy studies combined NAD-depleting drugs with chemotherapy and radiotherapy (Ekelund *et al.,* 2002; Progrebniak *et al.,* 2006), or combined PARP inhibitors with DNA-binding antitumour drugs as a suitable strategy in cancer therapy (Cepeda *et al.,* 2006). However, to this author's knowledge, no research to elucidate the consequences of low NAD levels on DNA repair, PARP activity and apoptotic cell death in CD38- expressing leukaemia cells, has been carried out to date. Therefore, according to the hypothesis that hyperactivation of PARP requires a certain level of cellular NAD to induce cell apoptosis and to decrease cell proliferation, it was postulated that in $CD38^+$ cells such as leukemia there will be a high demand for NAD that might render these cells more resistant to apoptosis.

The aim of the work described in this chapter was therefore to use a comet assay approach to investigate the response to DNA damage induced by UVB in cells expressing CD38 with low cellular NAD levels. In addition, the consequences of UVB on PAR production (confirmed by western blotting) and finally apoptotic cell death were examined. Whilst the data in this Chapter are preliminary, the results showed a significant level of UVB-induced DNA damage in cells with low NAD levels, in addition to cell resistance to apoptosis. Finally, these preliminary results may help to increase future understanding of the nature of cell resistance to apoptotic death in $CD38^+$ subsets of leukemia patients, even during chemotherapy or radiotherapy.

6.2 Material and methods

6.2.1 Materials

Low melting point agarose, normal melting point agarose, EDTA, Tris base, Triton X-100, DMSO, and Wright-Giemsa stain were all purchased from Sigma (Poole, UK). The PAR antibody was purchased from Abcam (Cambridge, UK).

6.2.2 Comet assay and quantification of DNA damage

Single-cell gel electrophoresis (the comet assay) is a microelectrophoretic technique for the direct visualization of DNA damage in individual cells. The comet assay (outlined in Fig. 6.1) was performed as described by Singh *et al* (1988) on HL60, RAJI and differentiated HL60 cells. Cell suspension (100 µl of 1 × 10^5 cells ml^{-1}) were transferred to individual 1.5 ml microcentrifuge tubes and centrifuged at 200 × g for 5 min. The supernatants were discarded and the pellets were each mixed with 85 µl of low melting-point agarose (0.5% in PBS), which was then pipetted onto agarose-coated microscope slides (pre-coated with normal melting-point agarose (1% in PBS) and dried at 37 °C). After the cell/ agarose mixture had solidified (4 °C for 15 min), the slides were then immersed vertically in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base, pH 10, containing freshly added 1% Triton X-100 and 10% DMSO) for 24 h at 4 °C. The slides were then washed three times vertically with neutralization buffer containing 0.4 M Tris-base, pH 7.5, and then placed into a horizontal electrophoresis apparatus (tank) filled with fresh, pre-cooled electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH 13.3). After 20 min of pre-incubation (unwinding of DNA), the electrophoresis was run for 20 min at a fixed voltage of 25 V and 300-400 mA, after which the slides were put in a tray and washed by adding neutralisation buffer in a drop-wise manner; this process was repeated

ten times. The slides were then left to dry at room temperature for 60 min. After drying, the slides were kept in a chamber in the dark at room temperature until analysis.

After 24 h, cells were stained with 20 µl ethidium bromide solution (2 µg ml^{-1}), and analysed at 200 × magnification with a Leica EL6000 fluorescence microscope (Bradford, UK). Assessment of DNA damage was based on the analysis of 100 randomly selected comets from each slide which were analysed by the comet IV imaging system (Perceptive Instruments, Suffolk, UK). In this system, tail DNA is considered to be the parameter most directly related to DNA damage. Each experiment was repeated three times with different cell preparations, and statistical analysis was carried out using a one-way ANOVA.

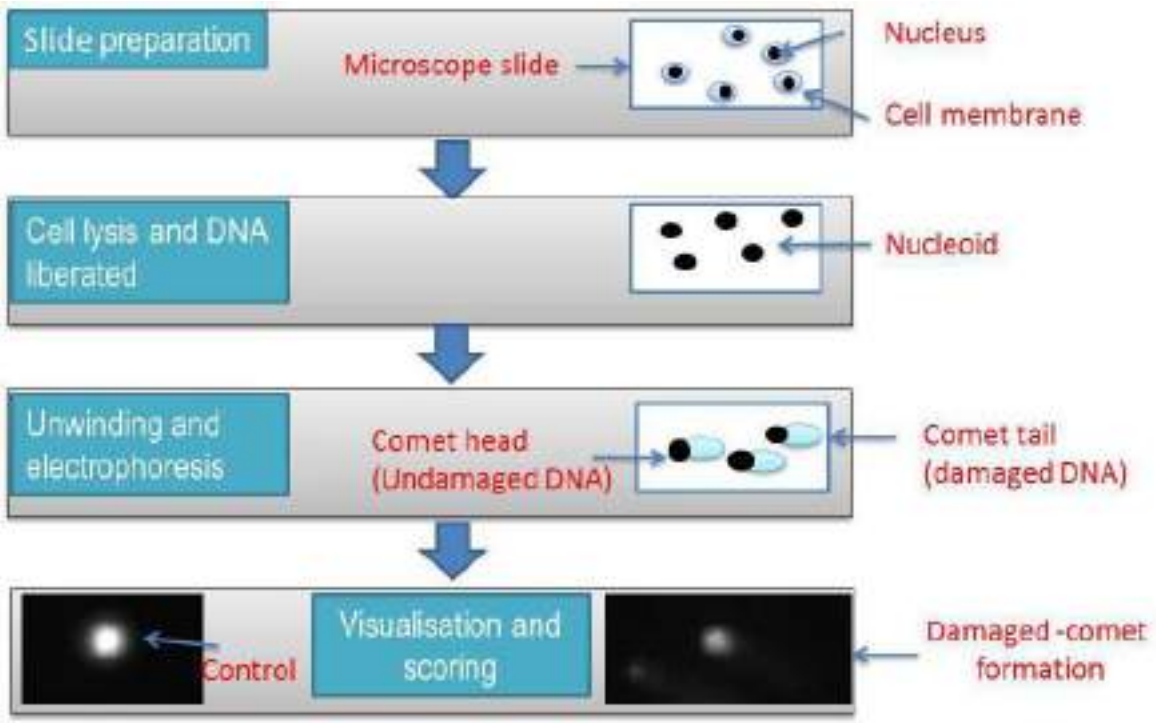


Figure 6.1 Schematic representation of the comet assay describing slide preparation, cell lysis, electrophoresis, visualisation and scoring steps (adapted from Tice *et al.*, 2000).

6.2.3 Validation of the comet assay under in vitro conditions using hydrogen peroxide

The comet assay was validated using hydrogen peroxide H_2O_2, as a reference genotoxic agent to determine oxidative DNA damage (Tice *et al.*, 2000). This was conducted by *in vitro* exposure of HL60 and differentiated HL60 cells to a range of H_2O_2 concentrations. The cell pellet was incubated with 100 µl of 0-300 µM concentrations of H_2O_2 for 10 min at 4 °C in the dark. Following the incubation, cells were washed with PBS to remove any remaining H_2O_2, and cell viability was determined using trypan blue. Slides were then prepared as described in Section 6.2.2 and processed for the comet assay.

6.2.4 UVB radiation of differentiated cells and induction of apoptosis

HL60, RAJI and 3 day differentiated HL60 cells were irradiated as freshly harvested suspensions. The cells were resuspended in PBS after two washes at the desired density (0.2 - 3 × 10^6 cells ml^{-1}). UVB irradiation was performed using a twin-tube lamp (TL-20W/12RS; Philips, Guildford, UK). UVB irradiation was calculated by using a Macam spectroradiometer (Macam SR9910, Livingston, UK) using integrated intensity between 280 - 340 nm (peak intensity of 310 nm). Irradiation was performed in 24-well plates. The UVB source was positioned directly above the cell suspension covered with a filter lid. The dose of UVB was 1.6 kJ m^{-2} for 560.8 s. In all experiments after exposure to UVB was completed, cells were immediately processed for the comet assay, MTT assay, Halo assay, Geimsa staining and western blotting (poly ADP-ribose modified protein production). In other samples measuring post-irradiation effects on DNA (DNA repair), cell suspensions were maintained at 37 °C for 45 min, 90 min and 6 h after exposure before processing using the comet assay etc. Cells used as

controls were similarly washed, centrifuged, counted, and placed in the 24 well plate, but were not exposed to UVB irradiation.

6.2.5 Halo-Comet assay

This technique was first described by Vinograd *et al.* (1965) and it was improved as the alkaline-halo assay by Sestili *et al.* (2006). In the normal comet assay, and in response to the electric current, charged DNA migrates away from the nucleus. The halo assay, in contrast, does not include an electrophoresis step. Also, the intact DNA remains within a residual nucleus-like structure called a nucleoid. If the nucleoid DNA contains strand breaks, a halo of DNA extends around the original form of the nucleus and may be visualized by fluorescence microscopy.

In this assay, following cell irradiation, the DNA damage was assessed as described in the Section on the comet assay (Section 6.2.2, except for omission of the electrophoresis step), and halo cells were visualized using a fluorescence microscope (Nikon Eclipse 80i, Surrey, UK), with image analysis software (NIS-Elements BR, Nikon, Surrey, UK).

6.2.6 Wright-Giemsa staining method

For morphological assessments of apoptosis, the UVB-exposed cells were stained using the Wright-Giemsa staining method. Cells were centrifuged and the pellets were resuspended in PBS. Cell suspension (100 µl of 1×10^6 cells ml^{-1}) were prepared on slides using a cytospin centrifuge (Shandon, Leicester, UK) and fixed with 5 µl methanol before staining with Wright-Giemsa stain. After 4 min, slides were rinsed in water and air-dried. The morphology of cells was examined under a light microscope (× 100; Olympus, Japan).

6.2.7 Statistical analysis

Statistical analysis of the data was assessed using Fisher's one way analysis of variance (Statview 5.0.1; Abacus concepts, USA) or Student's t-test as appropriate. Data are expressed as means ± SEM for three separate experiments in triplicate, unless otherwise stated. A difference of $P < 0.05$ was considered statistically significant.

6.3 Results

6.3.1 Effect of cellular NAD levels on UVB-induce DNA damage

The induction and repair of UV-induced DNA damage has been previously studied *in vitro* and in several cell lines (Cadet *et al.,* 1997; Ravanat *et al.,* 2001). However, the study in this Chapter aimed to induce DNA damage *in vitro* by using UVB irradiation, and also to investigate DNA repair in RAJI, HL60 and differentiated HL60 cells. The alkaline comet assay was used to detect the damaged DNA due to its high sensitivity (Collins, 2009). Among all the parameters provided by the comet software, the percentage of DNA in the tail (% tail DNA), is considered to be the most reliable parameter (Kumaravel and Jha, 2006), and was used in this work to represent cellular DNA damage.

To validate the comet assay, an experiment was conducted with H_2O_2 exposure to HL60 and 1 day ATRA-treated cells. DNA damage was clearly seen in the both groups of cells, and was similar in cases (Fig. 6.2). The percentage tail DNA data showed an increase in DNA damage with concentration in the range 30 to 300 µM H_2O_2. There was a ≈2.3 fold increase in percentage tail DNA with 300 µM H_2O_2 in HL60 and differentiated HL60 cells, compared to the untreated control. Thus, the comet assay proved to be a sensitive technique for the detection of DNA damage, and showed that DNA damage by H_2O_2 was concentration-dependent.

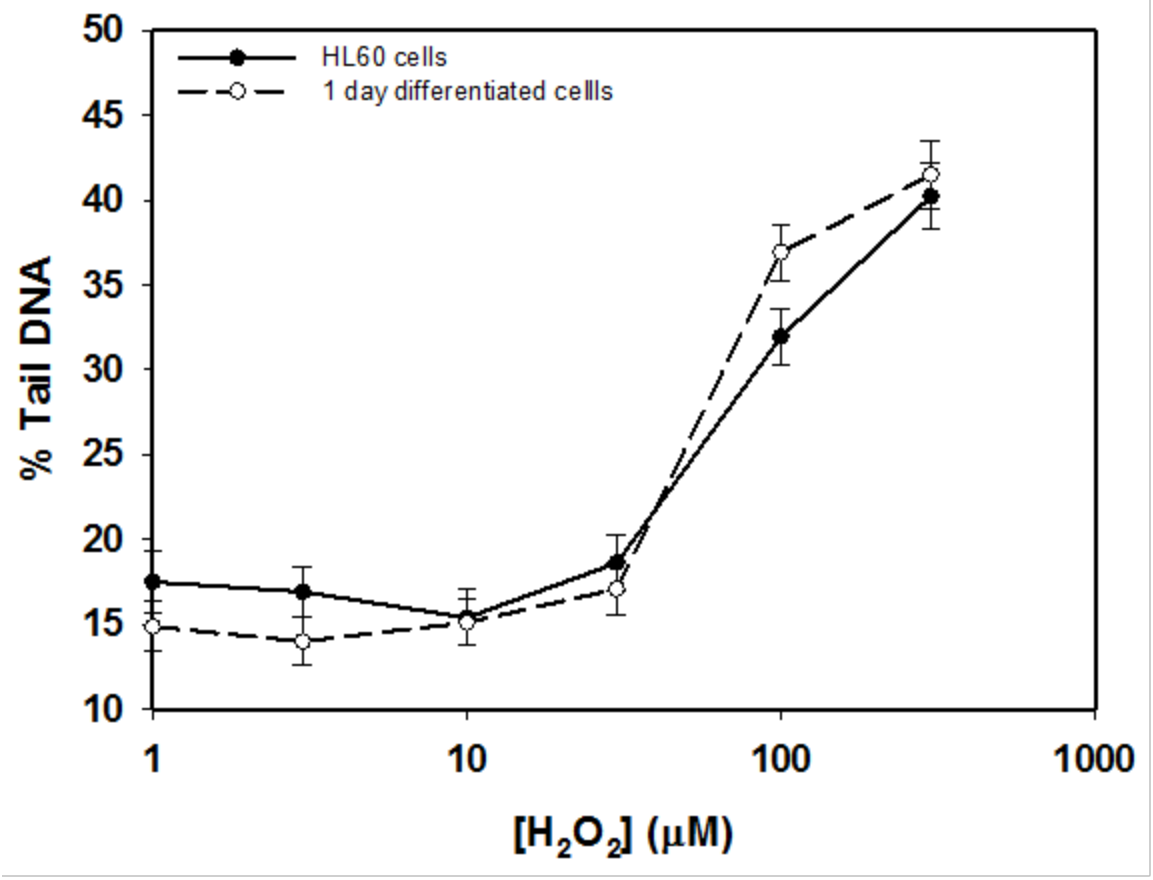


Figure 6.2 DNA damage expressed as percentage tail DNA in HL60 (solid line) and 1 day differentiated HL60 cells (dashed line) following 10 min *in vitro* incubation with different concentrations of H_2O_2 (1-300 µM). Data are means ± SEM, n = 1 (100 measurements).

In order to induce DNA damage, a UVB-irradiation system was used. A UVB meter was used to control the irradiation dose and to calculate the irradiation time. The emission spectrum from 280-340 nm of the lamp is shown in Figure 6.3 with a peak at 310 nm. Thus, to irradiate the cells with the desired dosage (1.6 kJ m^{-2}); the cells were irradiated for 560.8 s by using a twin-tube lamp (TL-20W/12RS; Philips). The irradiation time (t) was measured in seconds and was calculated using the formula: $D = I \times t$, where I is the UV radiation density (2.853 W m^{-2} {J s^{-1} m^{-2}} at 310 nm and D is dose of irradiation (1600 J m^{-2}).

In the current study, the induction of the DNA damage by exposure to 1.6 kJ m^{-2} UVB dosages was performed on the undifferentiated, 3 day-differentiated HL60 cells and RAJI cells. The results (Figure 6.4) showed that the UVB irradiation induced DNA damage in all cell lines, as estimated by percentage tail DNA. The DNA damage was more striking in differentiated HL60 cells and RAJI cells, in comparison to cells not expressing CD38 (undifferentiated HL60 cells), which showed relatively low levels of DNA damage. The same results were also confirmed in the comet images (Fig. 6.5). There was also no significant loss of cell viability (Figure 6.7) in any of the cell lines (cell viability using trypan blue exclusion was 70-80% in all cases). These results suggest that the maximum induced DNA damage can be clearly seen in cells that have low intracellular NAD levels ($CD38^+$ cells) than cells that have a high level of NAD.

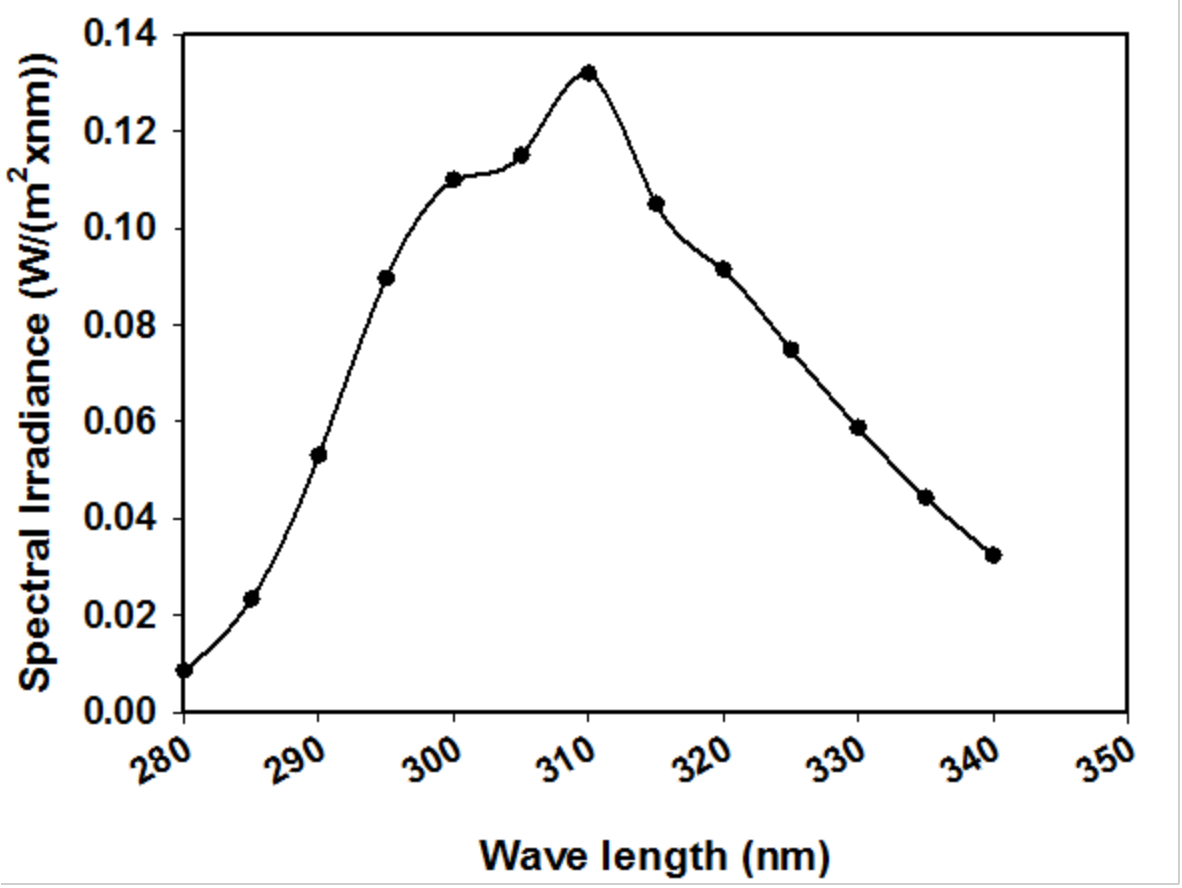


Figure 6.3 Spectrum of the twin-tube UV lamp with a maximum emission in the UVB region (310 nm). The lamp was used to irradiate the cells to induce DNA damage. The data represent a single measurement.

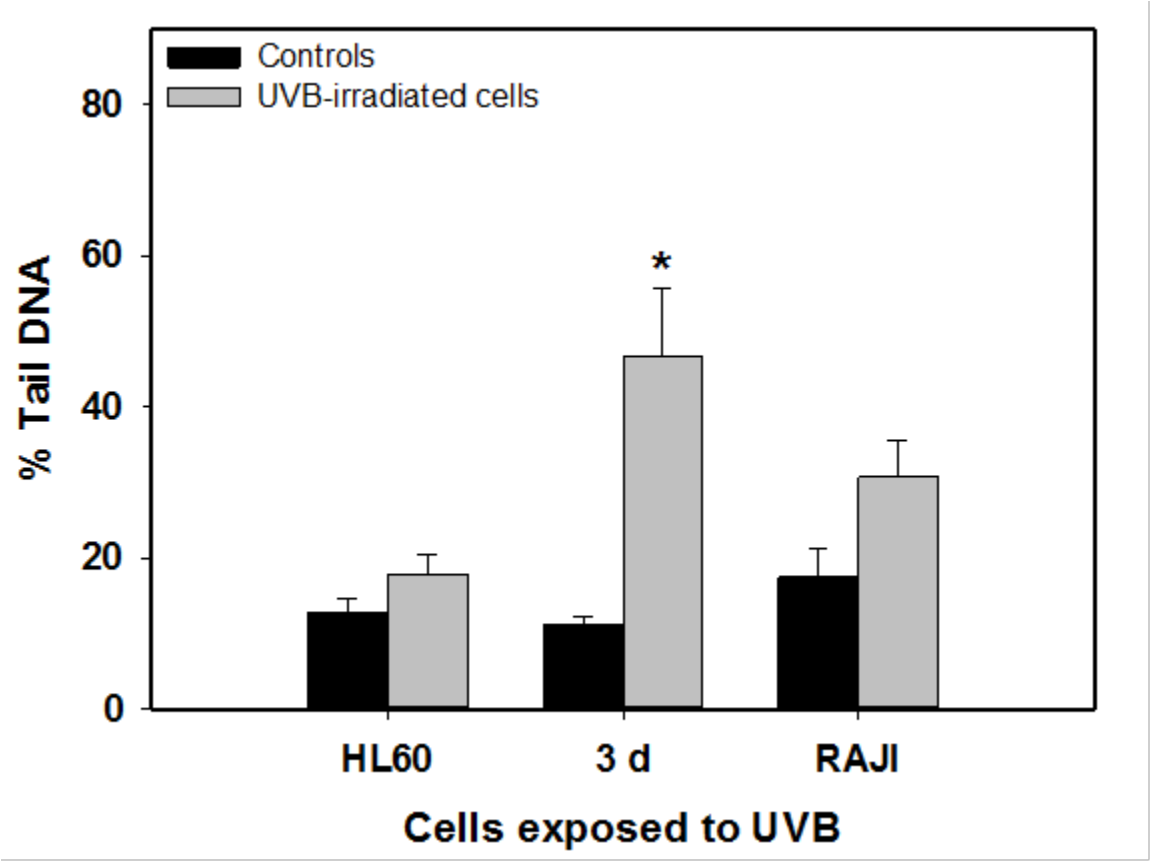


Figure 6.4 DNA damage expressed as percentage tail DNA in undifferentiated HL60 cells, differentiated HL60 cells (3 days) and RAJI cells following the irradiation with 1.6 kJ m^{-2} UVB. Data are means ± SEM, n = 3 (100 measurements per replicate). * denotes significant difference from the appropriate untreated control ($P < 0.05$).

The DNA damage was also assessed in undifferentiated HL60, RAJI and differentiated HL60 cells after recovery times of 45 min, 90 min and 6 hours after UVB-irradiation, to evaluate the DNA repair process (Fig. 6.6). There was an apparent drop in DNA damage in the all cell lines between 45-90 min after irradiation. This might suggest that DNA repair processes were more evident in HL60 and RAJI cells than in the differentiating HL60 cells. Interestingly, there was no decrease in cell viability at 45 min and 90 min in all cells after irradiation (Figure 6.7). Surprisingly, after a clear decline in the DNA damage at 45 and 90 min there was an increase in percentage tail DNA in all cells at 6 h post irradiation. No significant difference was observed between the different cell types at that time, which might suggest impairment in the DNA repair process. Moreover, cell viability results after 6 h recovery time showed a significant loss of viability (Figure 6.7) as determined by trypan blue exclusion in all of the cell lines.

Thus, the above results confirm that a decline in intracellular NAD in CD38 positive cells (3 days differentiated HL60 cells and RAJI cells) might increase the cell response to UVB-induced DNA damage as shown in Figure 6.4. However, these cells showed relatively recovered DNA after a short time of irradiation which might be related to PAR accumulation, which initiates the repair process in the case of moderate DNA damage (1.6 kJ m^{-2} dosages).

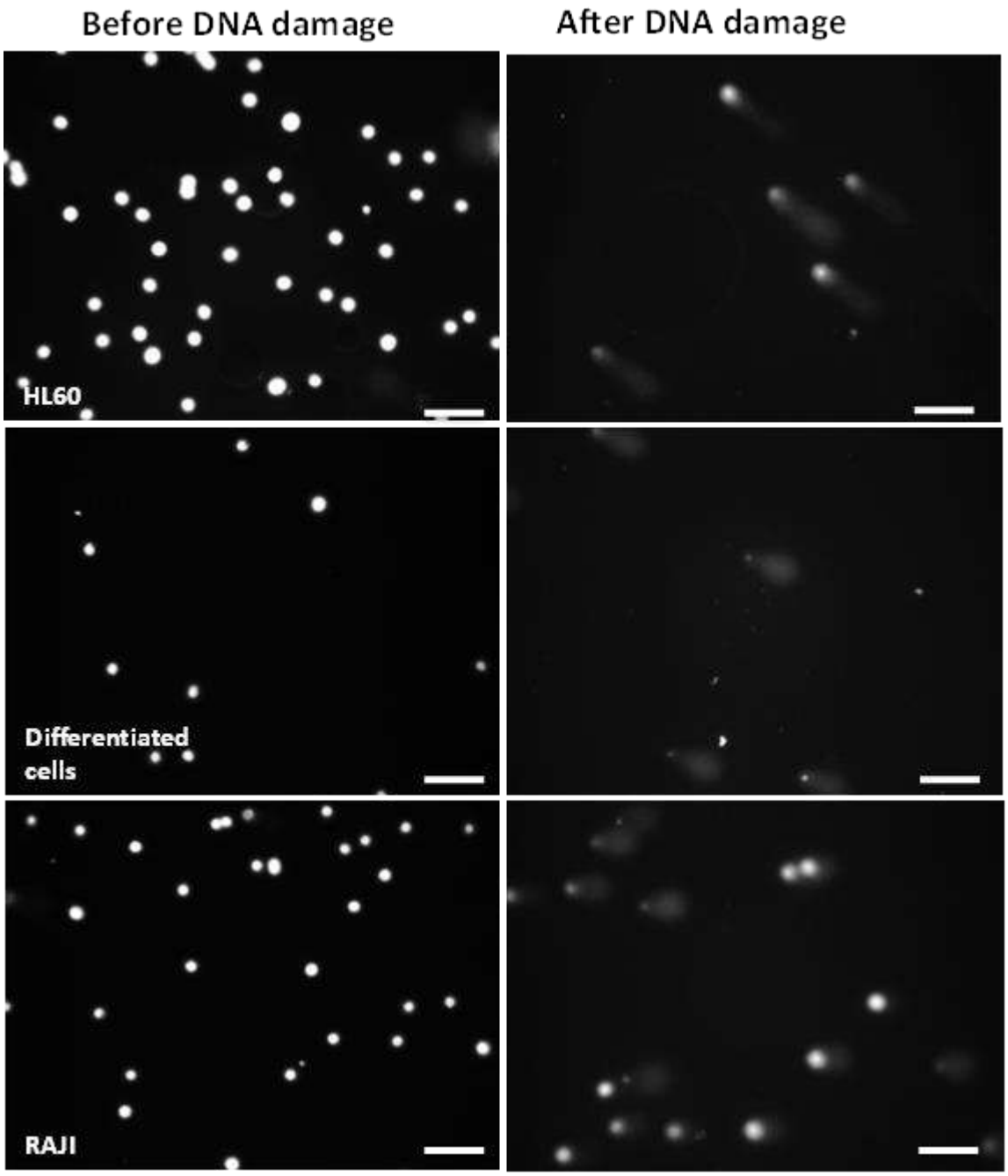


Figure 6.5 Representative comet images of undifferentiated HL60, 3 days differentiated HL60 cells and RAJI cells, which were exposed to UVB-induced DNA damage (1.6 kJ m^{-2}). Cells were stained with ethidium bromide before visualization. Magnification = ×200. Scale bars: 50 μm.

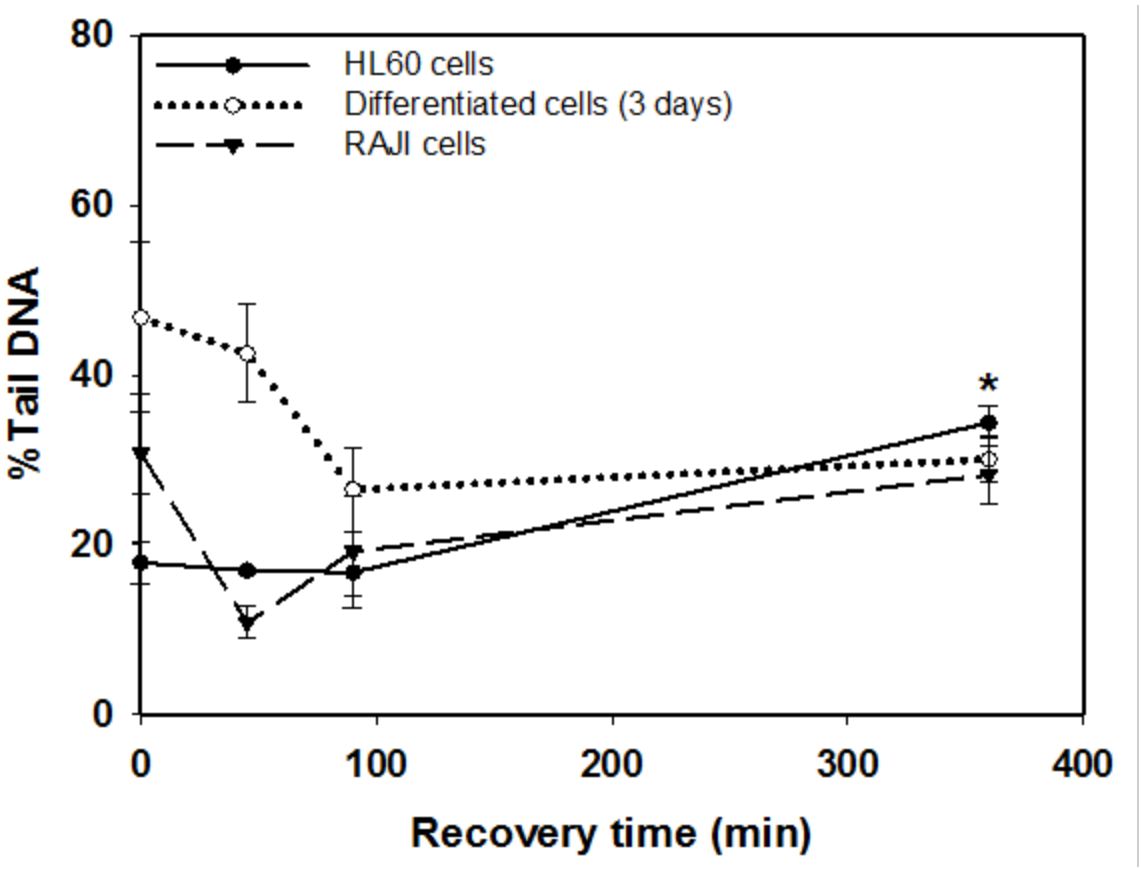


Figure 6.6 DNA repair of UVB-induced DNA damage (1.6 kJ m-2). Following HL60, ATRA-induced HL60 differentiation (3 days) and RAJI cell irradiation, the percentage of DNA damage was assessed after recovery times of 45 min, 90 min and 6 hours. Data are means ± SEM, n = 3 (100 measurements per replicate). * denotes significant difference from the appropriate control ($P < 0.05$).

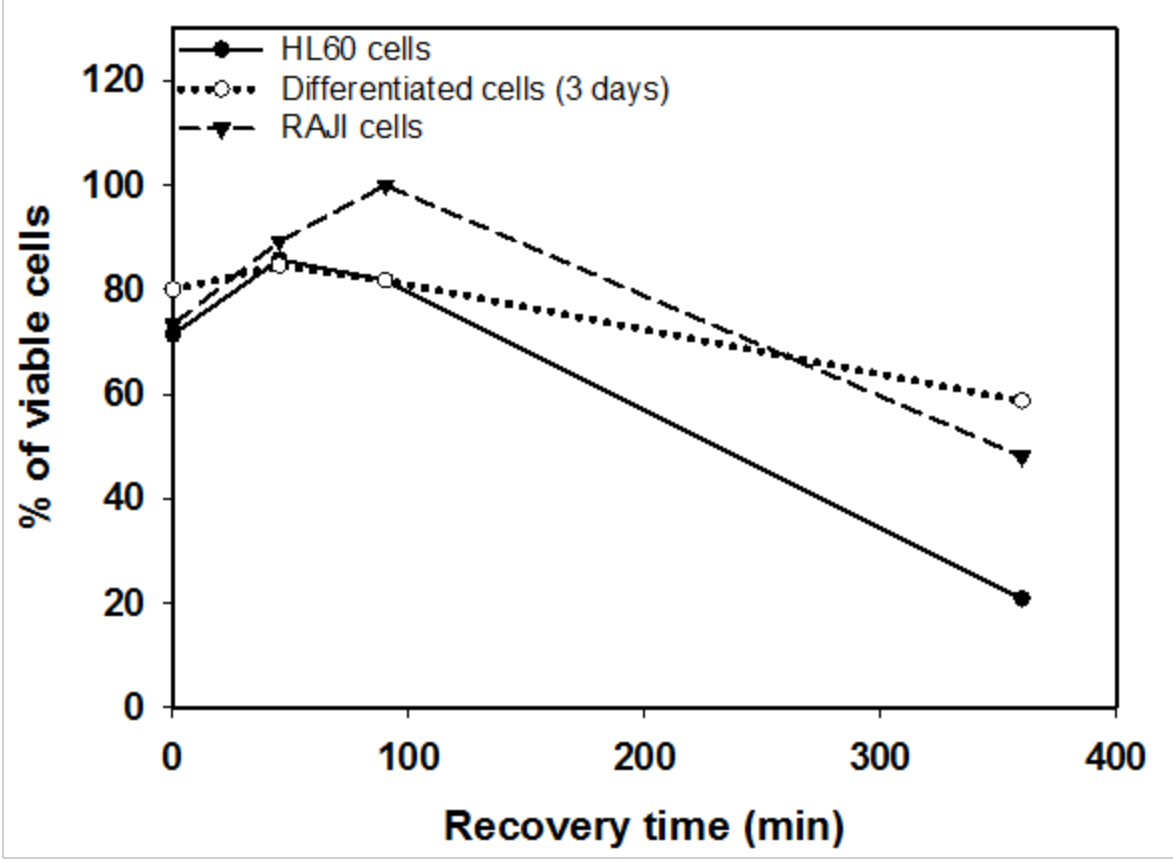


Figure 6.7 Cell viability following UVB-induced DNA damaged (1.6 kJ m-2) in HL60, ATRA-induced differentiated HL60 and RAJI cells as assessed by trypan blue exclusion over a recovery time of 45 min, 90 min and 6 h, n = 1 (4 measurements).

6.3.2 Effect of UVB on PAR production in CD38^{+}/ cells

Next, the effect of UVB on PAR accumulation in cells expressing CD38 was examined. It is well known that PARP-1 is activated upon binding to damaged or abnormal DNA (Durkacz *et al.*, 1980) and catalyzes the formation of poly(ADP-ribose) polymers (PAR) onto different acceptor proteins, including PARP-1 itself (auto PARsylation), using NAD^{+} as substrate. PAR accumulation is an immediate response following UV exposure. PARP catalytic activation was assessed by detection of its product, PAR-modified proteins, in CD38 negative cells (HL60) and CD38 expressing-cells (RAJI, 3 day differentiated HL60 cells) up to 6 h after UVB treatment (Fig. 6.8 A).

Western blotting (Fig. 6.8 A) showed that UVB exposure resulted in an observed decrease in PAR hypermodified proteins in HL60 cells, RAJI, and differentiated cells from 0-90 min post irradiation compared to the basal levels in each corresponding non-exposed control (Fig. 6.8 B), including PARP-1, whose modifications appear clearly between 100-200 kDa (Yu *et al.*, 2002). Interestingly, PAR production increases significantly at 6 h post-UVB treatment in all samples compared to immediately after treatment (Fig. 6.8 A); this was clearly shown in HL60 cells compared to the NAD-restricted cells (RAJI and differentiated HL60 cells) which showed less detectable PAR-hypermodified proteins (Fig. 6.8 A). The low PAR levels might reflect low availability of the substrate for PARP (NAD), probably restricted by CD38 activity.

Collectively, Western blotting results for poly ADP-ribose polymer expression might not show clear variations in cell response to DNA damage or PAR production which was based on whether or not the cells expressed CD38.

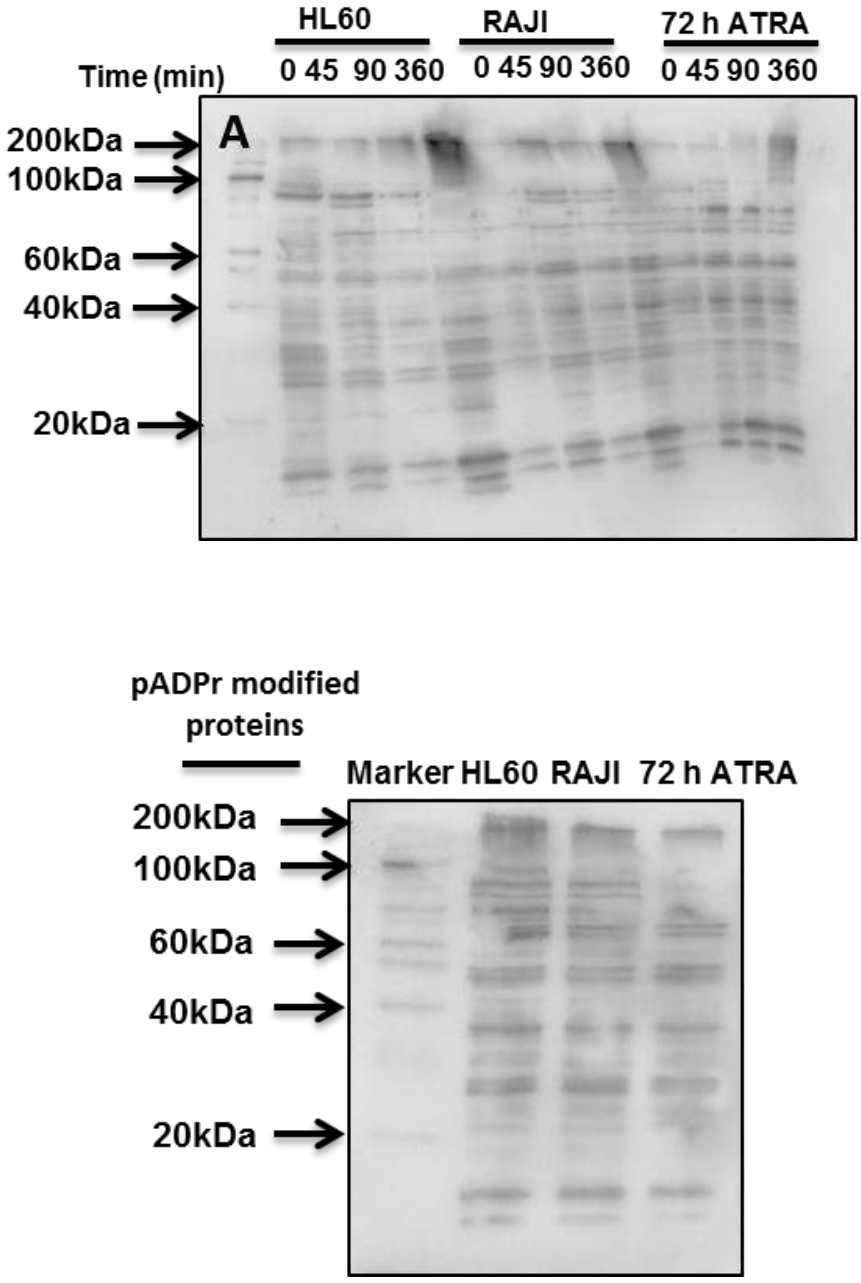


Figure 6.8 Western blotting analysis for poly ADP-ribose polymer expression under (A) normal conditions, represented by HL60 cells treated for 3 days with ATRA compared to the controls (RAJI and undifferentiated HL60 cells), and (B) PAR production after UVB exposure from 0-6 h for 50 µg cell lysate under reducing conditions and 12% SDS PAGE (Chapter 2, section 2.8.4). The figure represents one of two separate cultures.

6.3.3 Effect of UVB-induced DNA damage on apoptotic cell death in $CD38^+$ and $CD38^-$ cells

In this Section of the work, an attempt was made to examine a possible relationship between NAD levels, and effects on DNA-damage and cell death. In previous Sections, a different cell response to UVB-induced damage DNA was found. Therefore, it seemed important to further confirm these findings by using another method of assessing cell damage or death. Thus, two different morphological determination methods- the halo assay and Wright-Giemsa staining- were performed to compliment the data already obtained and to further investigate the effect of cellular NAD content on either DNA repair or apoptotic cell death. It is worth mentioning that radiation therapy is used in cancer treatment via the induction of DNA damage to kill cancer cells or to keep them from growing (Lawrence *et al.,* in: DeVita *et al.,* 2008).

Morphological observation of cell death was performed by two simple, sensitive, and reliable assays, the halo assay and Wright-Giemsa staining. These assays were used for the quantification of apoptosis in HL60, 3 day differentiated HL60 cells and RAJI cells that had been exposed to UVB as a DNA damaging source. Giemsa-stained cells (Fig. 6.9), and cells that were assayed by the halo assay (Fig. 6.10) showed morphological changes after UVB irradiation for up to 6 h. Typical apoptotic changes, including chromatin condensation, nuclear fragmentation and formation of apoptotic bodies, were clearly observed in Giemsa stained cells (Fig. 6.9). Those cells exhibiting morphological changes can be easily differentiated from normal cells which, under a light microscope, show normal morphology with no apoptotic bodies (Searle *et al.*, 1982). This was found in the controls for all cell lines. Furthermore, apoptotic cells assayed by the halo assay (Fig. 6.10) showed a halo of DNA with a hazy outer boundary that extends around the original form of the nucleus.

There was variation in cell responses to UVB-induced DNA damage that depended on the cell line and the time after irradiation. Thus, apoptosis appeared immediately after irradiation in all cell types as detected by Giemsa stain (Fig. 6.9) and halo assays (Fig. 6.10). Interestingly, HL60 cells showed a greater response to UVB-induced DNA damage with both assays at 0 h, compared to the other cells. Also, repair following irradiation was seen at 45 min and 90 min in HL60, 3 day differentiated HL60 cells and RAJI cells, respectively, and this observation might confirm initiation of the DNA repair process. No characteristics of apoptosis were seen for each non-irradiated control, HL60, differentiated HL60 cells and RAJI cells (Fig. 6.9, 6.10). Finally, after the recovery time, 6 h, apoptosis was observed in the differentiated HL60 cells and control HL60 cells to a greater degree than was observed in RAJI cells (Fig. 6.9, 6.10). These data appear to confirm that NAD is a key determinant of apoptotic cell death. Therefore, as NAD levels in HL60 cells are higher than those in RAJI cells, these cells were more response to UVB-induced DNA damage as confirmed by more apoptotic cell death. Importantly, similar results were obtained using the two assays (Fig 6.9; 6.10) and these morphological data are concomitant with DNA damage results (Fig. 6.6) for the same cells.

In summary, irradiation of HL60, differentiated HL60 cells and RAJI cells with UVB light caused the cells to undergo morphological changes characteristic of apoptosis, and the level of apoptosis differed between cells depending on the levels of CD38 expression, intracellular NAD content, PAR production and the time after irradiation. Collectively, these data supported the comet results and suggest that leukaemia cells that express CD38 might exhibit a resistance to apoptotic cell death that is induced under UVB radiation.

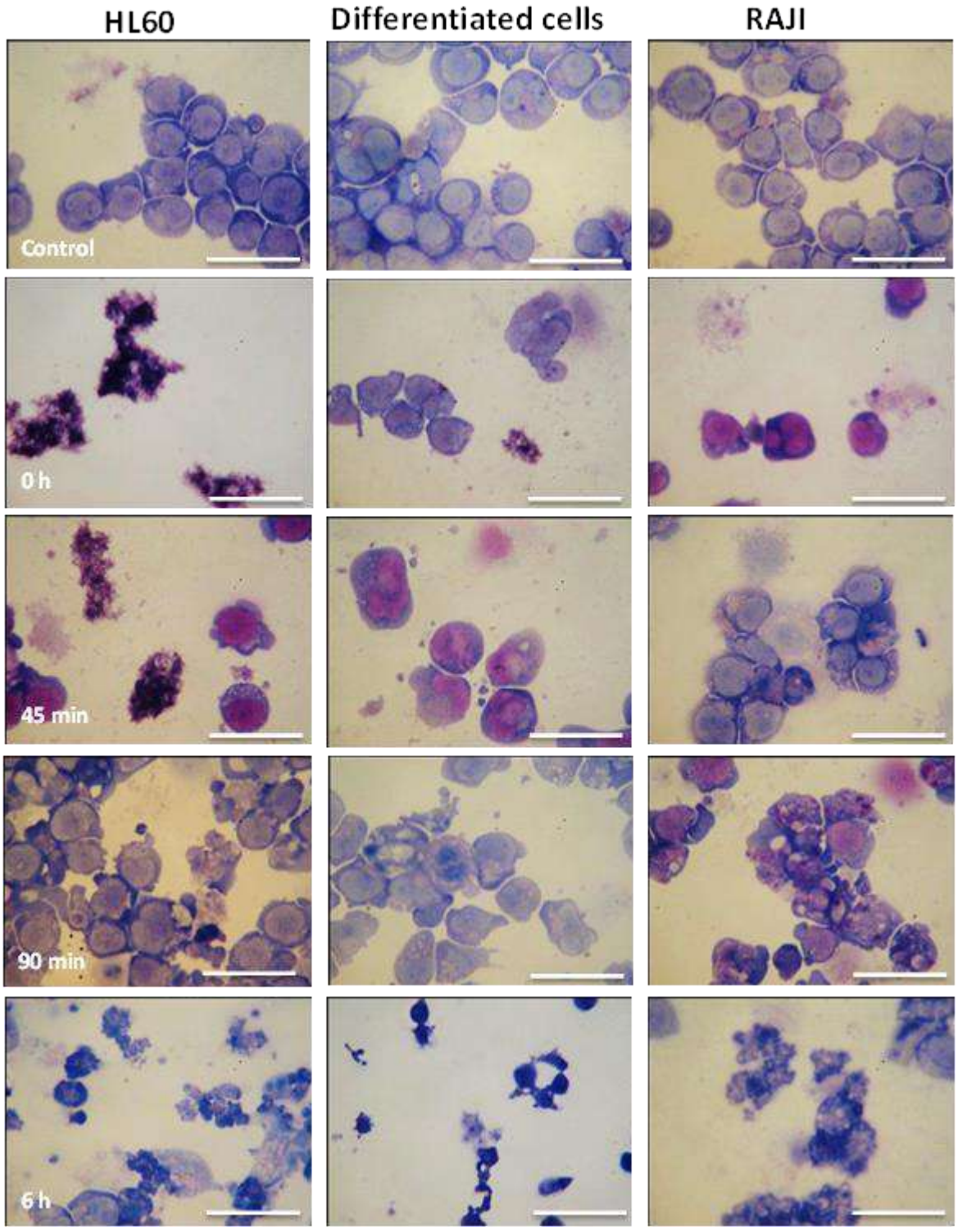


Figure 6.9 Photomicrographs showing HL60, 3 day- differentiated HL60 cells and RAJI cells stained with Wright-Giemsa stain before, and after irradiation (0-6 h). Cells exhibit features typical of apoptosis after UVB-irradiation. Original magnification x100. Scale bars: 50 µm.

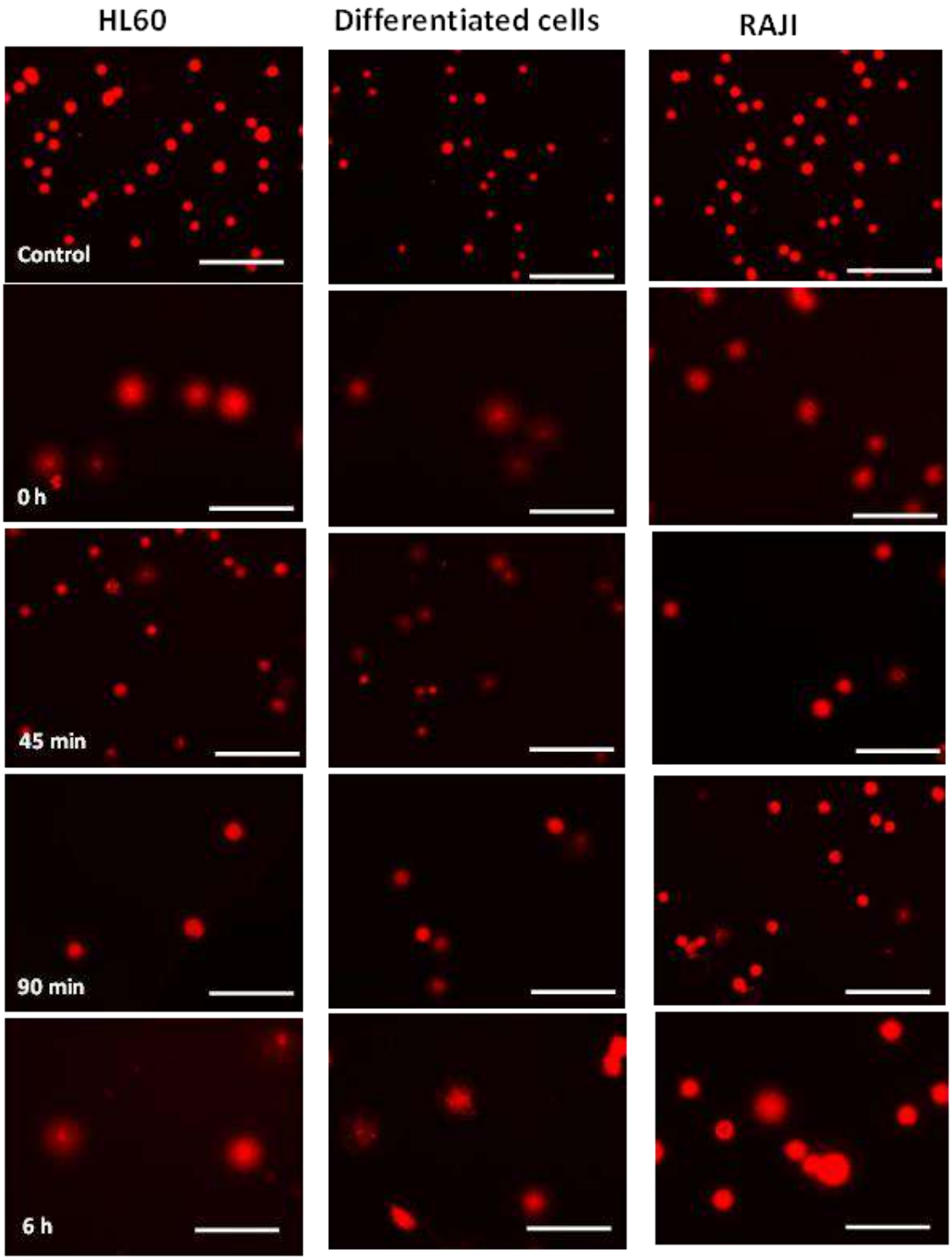


Figure 6.10 Photomicrographs processed during the halo assay showing HL60, 3 day differentiated HL60 cells and RAJI cells stained with ethidium bromide as controls or after UVB irradiation (0-6 h). Original magnification x 400. Scale bars: 50 μm.

6.4 Discussion

The aim of this study was to evaluate the effect of a CD38-mediated decrease in NAD levels on the cellular response to DNA damage (after UVB exposure), both in cells expressing CD38 (RAJI and 3 day differentiated HL60 cells), compared to undifferentiated HL60 cells, which do not express CD38. In the current study, the halo and comet assays were used to assess the DNA damage following UVB-induced DNA damage. Ultraviolet B (UVB) causes either direct DNA damage, forming cyclobutane pyrimidine dimers (CPD) and 6,4-photoproducts which are removed by nucleotide excision repair (NER), or oxidative DNA damage (for example single strand breaks), which are removed by base excision repair (BER), and single strand break repair (SSBR) (Caldecott, 2003). Interestingly, in order to repair the DNA damage, PARP is known to be activated, and it is implicated in both SSBR (Caldecott, 2003) and BER (Le Page *et al.,* 2003) of UV-induced oxidative DNA damage (Caldecott, 2003).

The UVB-induced DNA damage results shown in Fig. 6.4 clearly showed that CD38 negative cells (with lower intracellular NAD levels) showed less DNA damage compared to CD38 positive cells (RAJI and differentiated HL60 cells). Ultimately, these data confirm that the decline in NAD levels strongly enhances DNA damage. Furthermore, an important finding is that DNA repair was seen from the earliest times after irradiation as confirmed by the less DNA damage in HL60 and RAJI cells at 45 min and 90 min compared to differentiated HL60 cells. The results suggest that the cellular response to DNA damage is based on the variation in NAD levels. Although the differentiated cells expressed high levels of CD38 with lower NAD content, they are also showed low DNA damage at 90 min which might relate to the initiation of repair processes. A possible explanation for this repair is that the levels of NAD^+ were suitable to

initiate the repair process, and that NAD^+ might not drop to lower levels after PARP-1 activation. Also, PARP-1 activation may lead to rapid depletion of the cytosolic pool, but not of the mitochondrial pool, of NAD^+ (Ying *et al.,* 2005). These reasons might contribute to a relative DNA repair in the differentiated HL60 cells. Altogether, these results suggest that a repair process through PARP activation might occur in response to UVB-induced DNA damage, since at low or mild levels of DNA damage, PARP initiates DNA repair processes (for instance, recombination, remodelling of chromatin, transcriptional changes at the damaged site, and DNA base excision repair, Virag and Szabo, 2002). It is worth noting that an early PARP activation (represented by PAR accumulation) has been observed previously at 1 to 2 h after irradiation with UVB in mouse fibroblasts (Vodenicharov *et al.,* 2005). However, in the current study, early PAR production was observed in HL60 at 90 min after UVB-irradiation, increasing in all cell lines after 6 h. Surprisingly, at 6 h, a detectable level of DNA damage was also evident (≈30% tail DNA), and it was relatively similar in all cell lines. One possible explanation for these data is that PARP activation and PAR production might not be able to participate in the repair of UV-induced DNA damage, which might reflect the key role of NAD levels in the repair process. In addition to the effect of UVB irradiation in inducing DNA damage (Zong *et al.,* 2004), the decline in intracellular NAD, probably via CD38, might also participate in increasing this damage. CD38 might affect PARP activity by limiting the availability of NAD, ultimately mediating DNA damage and some of its consequences (collectively known as genomic instability, e.g. chromosomal aberrations, DNA translocations, deletions, and amplifications). In the absence of a repair system, probably PARP, genomic instability would rapidly accumulate and disturb DNA replication, gene expression and ultimately cellular and tissue homeostasis (Burkle, 2001).

NAD levels are an important factor in deciding cell fate. In general, two controversial hypotheses have been suggested involving NAD as a determinant of cell fate. It was firstly hypothesized that NAD depletion increased DNA damage (Winter and Boyer, 1973) and that PARP activation causes cell death via apoptosis (Yu *et al.,* 2002), necrosis (Ha and Snyder, 1999) or autophagy (Munoz-Gamez *et al.,* 2009). A second hypothesis proposed that NAD-depleted cells are resistant to apoptotic cell death, as demonstrated by a study of Wright *et al.* (1996). This study indicated that NAD-deficient cells are resistant to UV light-induced apoptotic cell death. Collectively, these two controversial hypotheses might explain that there are two mechanisms by which NAD-deficient cells respond when death appears imminent, these being either resistance or induced cell death. Importantly, the second hypothesis is strongly confirmed by the results of this Chapter. In the current study, it was hypothesized that the decrease in NAD levels might increase DNA damage and hence inhibits apoptotic cell death, as it was shown that cells with adequate levels of NAD undergo apoptosis, but that cells with restricted or lowered NAD levels show a relative resistance to apoptotic cell death. For example, apoptosis was more evident in HL60 cells than RAJI cells. It has been shown previously that UVB induced apoptotic cell death in HL60 (Lu *et al.,* 1996). The differentiated HL60 cells, unexpectedly, were also found to undergo apoptosis, which might be one of the consequences of ATRA-induced terminal differentiation of HL60 cells (James *et al.,* 1999). Thus, apoptotic cell death was NAD-independent in differentiated HL60 cells and NAD-dependent in both HL60 cells and RAJI cells. Altogether, cells that are expressing CD38 are expected to have an impaired repair system and accumulate DNA damage along with a characteristic resistance to cell death.

Several approaches have been developed to target NAD^+ metabolism for both the prevention and treatment of cancer (Jacobson *et al.,* 1999). For instance, pharmacological inhibition of PARP-1

activity worked as a suitable target to enhance the activity of antitumour drugs through inhibition of necrosis and activation of apoptosis (Southan and Szabo, 2003). Furthermore, researchers have developed drugs targeting the inhibition of NAMPT (the crucial enzyme in NAD synthesis that is overexpressed in human malignancies), that have anticancer properties through depletion of cellular NAD^+ (Van Beijnum *et al.,* 2002), such as using FK866 (Hasmann and Schemainda, 2003) and CHS828 (now under development as the prodrug GMX1777) either alone (Hjarnaa *et al.,* 1999) or in combination with cancer therapy producing DNA damage, the alkylating drugs and radiotherapy. For instance, FK866 has been combined with ionizing radiation in a mouse tumour model, which shows a delay in tumour growth, with no effect on normal tissues (Muruganandham *et al.,* 2005; Kato *et al.,* 2010). Importantly, the results of the current study might also be taken into account in cancer therapy while using chemotherapy or radiotherapy in combination with inhibitors of NAD biosynthesis, especially in $CD38^+$ leukemia subset patients.

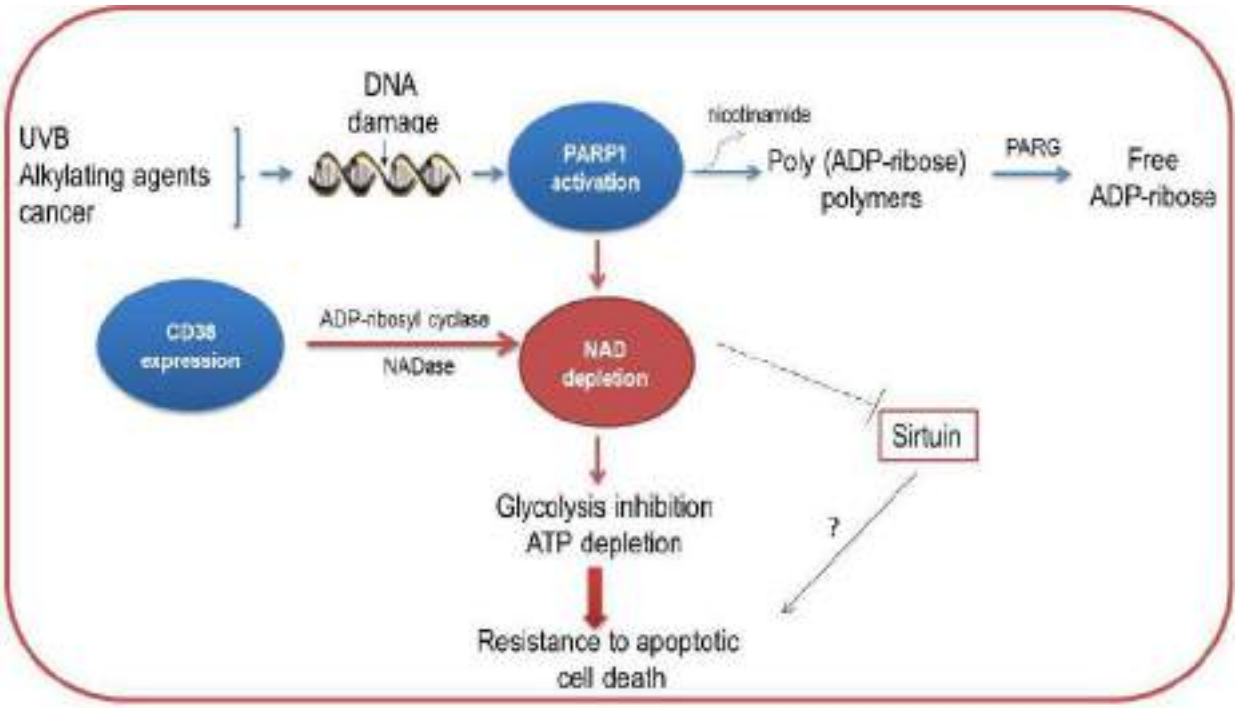


Figure 6.11 The effects of CD38 expression on NAD depletion and the role of NAD as a metabolic link between DNA damage induced by different stimuli or in cancer, and the resistance to cell death via a reduction in ATP levels.

In conclusion, NAD depletion in cells expressing CD38 might induce a resistance to apoptotic cell death and promote cell survival in leukaemia cell lines, which might have important implications for the pathogenesis and progression of cancer. In terms of leukemia patients, cells that are expressing high levels of CD38 (in patients with poor prognosis) would also be expected to show a similar relationship. Firstly, according to the results (Chapter 3), the increase in CD38 expression, concomitant with a decrease in NAD levels, will be linked with reduced glycolytic activity which may in turn affect ATP levels, since cells consume ATP to replenish NAD^+ (Virag and Szabo, 2002). Thus, a depletion of ATP might lead to increased resistance to cell death, since apoptosis is an energy-dependent process (Kass *et al.*, 1996), Secondly, as suggested by the

results presented in this Chapter, low intracellular NAD content may be as a result of CD38 activities, in addition to PARP activation (following DNA damage under UVB irradiation). CD38 activity and low NAD levels might further lead to restricted PARP activity, lowered PAR levels, reduction in the repair process and defective induction of apoptosis, since PAR is known as an inducer of apoptosis. This may lead to impairment of the repair system and failure to control the carcinogenesis.

Finally, the information presented in this chapter raises the possibility that in leukemia patients the combinations of chemotherapy or radiotherapy with CD38 expression might lead to poor responsiveness, drug resistance and a worsening of the disease stage. For this reason, DNA-damaging therapy that uses irradiation or drugs to destroy cancer cells is often accompanied by the development of drug resistance and severe side effects (Libura *et al.,* 2005; Mistry *et al.,* 2005). One possible explanation for this is that CD38 expression is an important regulator of intracellular NAD^+ pools and therefore of metabolic pathways that are related to the availability of NAD^+ rather than glycolysis, such as PARP reactions. This might reduce PAR production leading to a delay in apoptotic cell death in preference to cell resistance and survival. Altogether, combined CD38 expression with cancer therapy agents or radiotherapy may promote cell proliferation (Fig. 6.11), and drug resistance. For these reasons, $CD38^+$ patients at a more advanced stage of disease show poor responsiveness to chemotherapy and a shorter survival state in comparison to $CD38^-$ CLL patients (Morabito *et al.,* 2002). Thus, to improve patient therapy, one possible suggestion is to avoid the use of DNA-damaging therapy (chemotherapy or radiotherapy) in $CD38^+$ CLL patients, in order to reduce cell resistance to such treatment.

CHAPTER 7

GENERAL DISCUSSION

7.1 Discussion

Leukaemia is one of the common haematological malignant diseases, and CLL is the most frequent leukaemia in Europe and North America. It is a highly heterogeneous, incurable disease that ranges from a stable condition, not requiring treatment, to a rapidly progressive disease unresponsive to therapy (Hallek *et al.*, 2008). The presence of CD38 on the CLL cell surface has prognostic relevance, with high levels being associated with an unfavourable outcome (Damle *et al.*, 1999). For these two reasons, CLL was selected as a disease model in the current study to investigate the underlying hypothesis. Generally, most studies to date have focused on the role of receptor function of CD38 in poor CLL prognosis. This study is the first to describe an important role of CD38 enzymatic function in altering NAD levels and of their contribution to leukaemia development and progression, in addition to its receptor function. The human leukaemia cell line HL60, was predominantly used as an alternative model for leukemia in the current study as a model expressing CD38 when differentiated to neutrophil-like cells using ATRA. The current findings suggest that CD38 expression plays a key role as a determinant of cell survival, which is mediated through consumption of NAD, as a redox cofactor and the substrate for a network of NAD-consuming enzymes. Overall, these findings, combined with previous findings, lead to three hypotheses describing the synergistic connection between the receptor and enzymatic functions of CD38, which might help to explain the poor prognosis in $CD38^+$ leukemia subset patients.

Initially, Vaisitti *et al.* (2011) described two hypotheses in terms of the mechanism by which CD38 regulates the homing process and therefore why patients with $CD38^+$ CLL clones experience a generally more aggressive disease and a worse prognosis. In the first hypothesis, it

was suggested that CD38 could transmit its own indirect signals through the generation of Ca^{2+} active messengers (Vaisitti *et al.,* 2010). Extracellular CD38 catalyses NAD^{+} hydrolysis, generating ADPR and, at lower levels, cADPR. These two messengers are transported inside the cell by CD38 itself or by active channels, where they could then trigger Ca^{2+} influx from the binding to TRPM2 or RyR (Perraud *et al.,* 2001). An increase in Ca^{2+} concentrations may lead to direct activation of Ca^{2+}-sensitive tyrosine kinases (Schaller, 2010), in addition to direct nuclear translocation of Ca^{2+}-sensitive transcription factors (Graef *et al.,* 1999). Ultimately, this leads to the initiation of a transcriptional program regulating proliferation.

The second hypothesis suggests that CD38 induces the formation of highly stable supramolecular complexes that include surface molecules as well as intracellular signalling adaptors (Deaglio *et al.,* 2007). CD38 plays direct role as a molecular amplifier that activates the polymerization of actin and nuclear events, specifically in the presence of its non-substrate ligand CD31. In addition, the presence of other possible molecules as previously described (Chapter 1) together creates a suitable environment for CD38 to work as a master regulator of the CLL cell homing process in patients with $CD38^{+}$ CLL clones.

The current study explores a third hypothesis (Fig. 7.1), which postulates that, in addition to the aforementioned role of CD38 as a receptor, CD38 enzymatic functions are also involved in CD38-mediated poor prognosis in leukemia patients. Tests of the current hypothesis had two major aims, the first was to provide solid confirmation that CD38 is the main NAD consuming enzyme, but not its analogue (CD157), nor other NAD consumers (PARP or sirtuin). The findings (Chapter 3) supported the hypothesis, confirming the major role of CD38 in altering NAD levels to the lower levels concomitant with its expression; this relationship was reversed by

the impact of kuromanin in inhibiting CD38 cyclase activity. This study produced results which corroborate the findings of Barbosa *et al.* (2007), who demonstrated that loss of CD38 in CD38 KO mice had a major effect on NAD homeostasis, since a significant boost in NAD levels were shown.

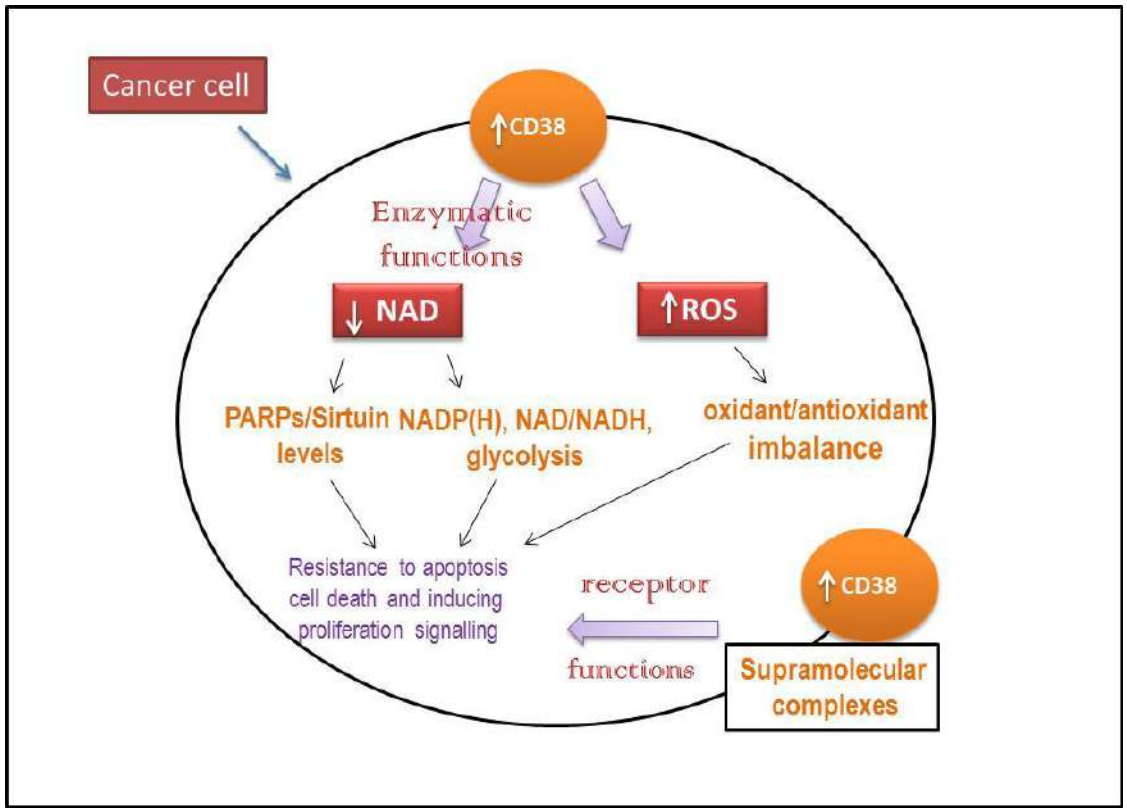


Figure 7.1 Schematic diagram representing the third hypothesis for the functional role of CD38 enzymatic functions mediated by NAD, combining with its receptor functions in inducing cell proliferation and poor prognosis in $CD38^{+}$ leukemia patients.

The second aim in testing the current hypothesis was to investigate the consequences of CD38 enzymatic function (since it has a major role in NAD consumption) on NAD-dependent processes, cell physiology and induction of cell proliferation in leukaemia, specifically in chronic lymphocytic leukaemia. The results in Chapters 4 and 6 provide positive data that support this hypothesis. The results confirmed that many processes were affected by CD38-mediated lowering of NAD levels. For example, glycolytic activity, which was reflected by reduced lactate production and a relatively constant NAD^+: NADH ratio. This might directly affect cellular energy as the NAD^+: NADH ratio is a measure of the energy status of a cell (Ying, 2006). Moreover, CD38 activity might also restrict NAD-dependent enzyme activities (PARP, sirtuin) and their dependent processes in the cells by limiting the substrate availability (NAD). These enzymes mediate important roles in modifying cellular functions such as genomic stability, apoptosis, cell signalling and stress tolerance (Malavasi *et al.,* 2010). CD38 expression was accompanied by elevated lipid peroxidation (as assessed by TBARS) and low total glutathione levels, which represents the antioxidant status in the cells, suggesting that an imbalance between oxidant/antioxidant status was concomitant with CD38 expression. Collectively, these two results using differentiated HL60 cells support the third hypothesis.

It is worth noting that ATRA-induced CD38 expression in HL60 cells has been shown to induce cell apoptosis (Mehta *et al.,* 1996), despite a large decrease in intracellular NAD levels. One of the possible explanations for this is that ADP ribosylation of nuclear CD38 may trigger an induction of apoptosis in ATRA-treated HL60 cells (Yalcintepe *et al.,* 2005). Similar results have been obtained *in vivo* to the effect of ATRA, in patients with acute promyelocytic leukaemia, but with a side effect known as an APL differentiation syndrome (Sanz *et al.,* 2009). In other cells, however, CD38 has been attributed to different effects on cell growth; its cellular

effects have shown to be ambiguous, since CD38 expression was either a positive or negative regulator of induced cell differentiation and growth arrest, depending on the expression level per cell (Lamkin *et al.,* 2006). For example, in 3T3 or HeLa cells, it promotes cell cycle progression (Zocchi *et al.,* 1998), in B lymphocytes or T lymphocytes it can cause apoptosis (Kumagai *et al.,* 1995; Tenca *et al.,* 2003), but in CLL, a B cell leukaemia, CD38 is an indicator of poor prognosis (Ghia *et al.,* 2003). According to previous studies, $CD38^{+}$ CLL clones show a resistance to apoptosis; this might be due to a decline in NAD levels that occur while CD38 expression is upregulated. Hence, from the current data in differentiated HL60 cells, it is possible to suggest that leukemia cell survival might not be affected by NAD-depletion. Instead, cancer cells with low cellular ATP levels may show higher resistance to programmed cell death due to the high energetic requirements of apoptosis. The findings relating to the current hypothesis along with the previous hypotheses may provide a better understanding of the mechanism of resistance of leukemiacells to apoptosis.

In cancer cells, the same basic metabolic pathways are utilized to generate energy as in normal cells, but some changes in a tumour microenvironment lead to protective metabolic adaptation (DeBerardinis, 2008; Semenza, 2008). For example, the cells in such an environment tend to use lactic fermentation in the absence of oxygen, which is a faster way to generate metabolic energy (Jones and Thompson, 2009). The main advantage of lactic fermentation is that ATP can be obtained at a faster rate through a simpler process (DeBerardini, 2008). Hence, cancer cells exhibit a high rate of NAD turnover due to a high glycolytic activity in addition to high levels of ADP-ribosylation activity (Gagne *et al.,* 2006), resulting from PARP activation (required for DNA repair and genome stability). Thus, in cancer cells, low intracellular NAD levels may lead to a resistance to apoptosis and induce cell proliferation. Interestingly, in CD38 negative

leukaemia cells one might expect that the replenishment of NAD levels might relatively enhance the DNA repair process and control disease progression. However, the same effect might not be seen in leukaemia cells that express CD38, since NAD-depletion would have more impact on NAD-dependent process and cell survival. Thus, low NAD levels lead to a drop in ATP, the level of which is an important factor in the process of apoptosis. Moreover, the metabolic pathways that depend on NAD availability such as sirtuin and PARP would also be affected. Low intracellular NAD might lead to accumulation of damaged DNA that would further decrease NAD levels due to PARP hyperactivation. Altogether, a severe drop in NAD levels leads to a similar drop in the determinant factor of apoptotic cell death, ATP. Ultimately, these environments create cellular resistance to apoptosis, and also facilitate the cell proliferation process. It is worth noting that drug resistance in tumour cells is a common obstacle in cancer chemotherapy. Resistance includes decreased drug accumulation, intracellular drug detoxification, enhanced DNA repair/ tolerance and failure of apoptotic pathways (Fuertes *et al.*, 2003). Beyond NAD depletion, it has been suggested that highly resistant tumour cells may express different versions of caspases or they may contain endogenous caspase inhibitors that limit apoptotic cell death pathways (Schimmer *et al.*, 2003).

In conclusion, targeting CD38 enzymatic functions in leukemia therapy along with its receptor function might serve as a possible solution to apoptotic cell resistance or poor cell metabolism in leukemia patients, and that future studies should pay attention to the evaluation of intracellular NAD levels as well as CD38 expression levels as a marker for poor prognosis in leukemia patients.

7.2 Future studies

One of the current treatments for leukaemia is a drug-based chemotherapy that uses one or more drugs to destroy cancer cells and induce apoptosis. However, this may be accompanied by the development of drug resistance and severe side effects (Libura *et al.,* 2005; Mistry *et al.,* 2005). Chemotherapeutic alkylating agents have been shown to cause miscoding lesions, chromosomal aberrations (Veld *et al.,* 1997) and secondary cancer, particularly leukaemia. They may also depress NAD^+ levels (Dreizen *et al.,* 1990). For instance, the chemotherapeutic agents (e.g., 5-fluorouracil, 6-mercaptopurine) interfere with the conversion of tryptophan to niacin (Stevens *et al.,* 1993). Moreover, rat studies have shown that niacin deficiency significantly increases the risk of chemotherapeutic induced secondary leukaemia (Kirkland, 2003). Therefore, it is imperative to develop other potential therapeutic agents for the treatment of this disease. Thus, NAD metabolism has an important position in total cellular metabolism, because it has multifunctional roles as a cofactor for individual enzymes and as a substrate for NAD^+-consuming enzymes. It could pose an attractive target for the treatment of various pathologies, especially in the fields of CD38 biology, linked to the prevention of aging and its related diseases like obesity and cancer. The results obtained in the current study might successfully be applied in the field of cancer therapy and also open the door for future studies to further characterize the functional role of CD38-mediated NAD depletion in the development of leukaemia, for example if the observations obtained from a current study using the human leukaemia model (HL60) are confirmed in the context of leukaemia cells. If so, therapeutic strategies which target this CD38/NAD relationship, such as investigating the effect of manipulating NAD levels on prognosis in $CD38^+$ leukemia patients, might be effective in fighting leukemia. However, several areas still need further investigation to clarify the precise effect of CD38 expression on the

nucleotide levels or on other NAD-dependent reactions. One of the important questions to be addressed is the levels of NADP in this situation; cADPR levels also need to be measured while NAD is consumed. If levels of this messenger decrease concomitantly with NAD levels, this raises further questions as to how CD38 induces proliferation signalling since, as previously mentioned, cADPR plays a role in the control of cell proliferation via Ca^{2+} signalling (Zupo *et al.,* 1994; Hardingham *et al.,* 1997). Similar investigations need to be focus on NAADP as a second messenger produced from its precursor, NADP.

Moreover, according to the results obtained in Chapter 5, CD38 expression can be downregulated by adding NAD^+ to the medium, or using FK866. It would therefore be tempting to investigate the mechanism of this inhibitory effect as a future study. It has previously been observed that in cancer cells DNA damage can stimulate NAD^+ biosynthesis (Jacobson *et al.,* 1999), through upregulation of the expression of NAMPT (Van Beijnum *et al.,* 2002), which is the rate-limiting enzyme in the salvage pathway from the breakdown product nicotinamide (Revollo *et al.,* 2004). That suggests that NAMPT may be crucial in maintaining cellular NAD^+ levels in tumours. For that reason FK866, a potent inhibitor of human NAMPT, is used, and the consequent reduction in NAD^+ levels has been seen to induce apoptosis of tumour cells (Hasmann, and Schemainda, 2003; Muruganandham *et al.,* 2005). If a similar mechanism applies to the primary leukaemia cells ($CD38^+$ cells), and according to the results in Chapter 5, then FK866 might serve as a useful therapeutic agent target for leukemia patients. This suggested study might succeed in the case of moderate DNA damage in cancer cells, since depletion of NAD levels and also CD38 expression by using FK866 might be a useful tool to induce apoptotic cell death. Interestingly, downregulation of CD38 expression might reduce its signalling effect on proliferation. Furthermore, it would be interesting to determine whether the

same process could apply to leukaemia patients by using NAD^+ supplementation, since applying NAD^+ has also been shown to downregulate CD38 expression (Chapter 5). An elevation of intracellular NAD levels through control of NAD homeostasis pathways, either via consuming enzyme pathways, or recycling pathways could heavily impact on metabolism, cellular viability and signalling pathways. In this situation, repletion of cellular NAD levels by adding NAD^+ directly to the culture medium with concomitant inhibition of CD38 expression might also lead to apoptotic cell death, since NAD levels are a determinant of cell survival.

CD38, as a major NAD consumer enzyme in the cells, has developed from a mere marker to a disease modifier in leukemia. Thus, it is important to determine the role of other NAD consumers in modifying the environment or inducing proliferation signalling in cancer cells. Previously published studies observed that inhibition of PARP-1 in cancer cells exposed to DNA-damaging drugs would decrease DNA repair and would induce apoptotic cell death, and may also increase the sensitivity of tumour cells to DNA damaging antitumour drugs (Munoz-Gamez *et al.*, 2005). Interestingly, this was more effective against tumour cells than against normal cells. However, in cancer cells that express CD38 the mechanism may be different, since NAD would be consumed by CD38 as the main NAD-degrading enzyme rather than PARP, and this might delay apoptotic cell death according to the results Chapter 6. Thus, utilizing CD38 inhibitors along with PARP inhibitors might elevate intracellular NAD levels and hence might also target apoptotic cell death. Furthermore, in the case of severe DNA damage combining intracellular NAD-elevating compounds (e.g. nicotinamide or NAD^+) along with CD38 inhibitors and PARP inhibitors might serve as an excellent therapeutic approach for $CD38^+$ leukaemia patients, since elevation of intracellular NAD levels might help to induce apoptotic cell death. Indeed, the fact that NAD^+ levels could be elevated by selective inhibition of NAD^+ consumers, PARPs or CD38 might lead

to the activation of other NAD^+ consumers, such as sirtuin. The latter enzyme has important functions such as gene silencing, longevity and genome stability (Zhang, 2003; Pillai *et al.,* 2005). Nevertheless, blockage or inhibition of one pathway of NAD^+ consumption might cause potential side effects, as, for example, it has been suggested that SIRT1 and 2 are crucial antiapoptotic molecules in leukaemia cells and have a role in the development of cancer; thus, the SIRT inhibitor, sirtinol, effectively induced cell death and that may be a useful therapeutic agent for leukaemia (Peck *et al.,* 2010). Therefore, further studies are still required to evaluate whether such a strategy may be of therapeutic value, or whether utilizing inhibitors of DNA repair pathways concomitant with the above suggestions could be an efficient strategy for cancer therapy.

One of the fundamental problems of tumour cells is the phenomenon of lactic fermentation; the metabolic adaptation of cancer cells in response to hypoxia has been shown to be associated with reduced sensitivity to common anti-cancer agents (Xu *et al.,* 2005). Thus, it has previously been suggested that targeting the glycolytic pathway might preferentially sensitize cancer cells to chemotherapeutic agents without significant toxicity to normal cells (Xu *et al.,* 2005). Hence, in combination with the results in Chapter 5, CD38 expression has been seen to be upregulated under hypoxic conditions (specifically 2% O_2). Therefore, it is worth investigating the role of CD38 in inducing cell proliferation under the same *in vivo* conditions by exposing the cells to a range of oxygen levels in order to characterize the enzymatic and receptor functions of CD38 in a hypoxic environment similar to that of cancer cells.

Moreover, it is interesting to note that recently published studies by Vaisitti *et al.* (2011) found that CLL cells taken from patients died easily in culture and appeared to have severe

impairments in vital signalling pathways. One possible explanation is that CLL cells from patients are more dependent on the external environment and supporting signals from other cells *in vivo* (Vaisitti *et al.,* 2010). This reflects a key role of the host environment in CLL progression and suggests that targeting the host might be a valuable therapeutic target. For instance, In addition to targeting CD38-mediated NAD depletion and its related processes, it would be interesting to target the non-substrate ligand for CD38, CD31. leukemia cells' resistance to apoptosis is probably due to CD38/CD31 interaction-mediated anti-apoptotic signals. Thus, targeting CD31/CD38 interaction and their proliferation signalling might also be a potential target in leukaemia, since CD38 receptor functions have a large signalling effect on proliferation in addition to its enzymatic functions.

Finally, it would be interesting to investigate the role of CD38 expression leading to depletion of intracellular NAD and the consequences of this relationship in the development of other diseases beyond leukaemia such as obesity. CD38 expression is linked to obesity and it has been suggested CD38 deficiency has a key role in preventing the development of obesity following NAD elevation (Baur *et al.,* 2006). Thus, future studies might target NAD levels through regulation of CD38 expression, such as combining CD38 inhibitors with NAD-elevation compounds which is also found to down regulate CD38 expression, as shown in the current study.

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Appendices

Enzymatic activity of CD38 comparative to NAD levels in leukemia cells

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Background

Human CD38 is a cell surface glycoprotein expressed in a wide variety of cell types that has both enzymatic and receptorial functions. As a receptor, CD38 controls signaling pathways involved in the activation, growth, and survival of lymphoid and myeloid cells. As an enzyme, CD38 uses NAD(P) as a substrate to form a number of biologically active compounds including cADPR, NAADP and ADPR (1) although the major physiological role would seem to be in control of NAD levels. Such control has wide ranging implications on cell physiology not only due to modulation of basic metabolism but also as NAD has recently been identified as a substrate in a variety of signaling reactions.

CD38 and its metabolites have been proposed to be involved in a number of human diseases ranging from Diabetes to HIV infection. CD38 is also a widely used negative prognostic marker (2) in chronic lymphocytic leukemia (CLL) where increased CD38 expression correlates with a worse prognosis.

While much is known about how the receptor activity of CD38 contributes to the CLL effects, the enzymatic activity has not been studied.

Objectives

- To understand the relationship between CD38 expression and NAD levels.
- To understand how this relationship may influence the physiology of CLL cells

Methods

- RAJI cells were used as CD38+ cells. HL-60 (CD38-) were treated with all trans retinoic acid (ATRA;1 µM) to induce differention to neutrophil-like cells.
- ADP-ribosyl cyclase activity of CD38 was measured using the fluorescent NGD assay. The initial rate was monitored by measuring the rate of formation of cGDPR (3).
- NAD was extracted and it levels were determined using a modification of the protocol described by Leonardo *et. al.* (4).

Results

CD38 is expressed during differentiation

HL-60 cells were stimulated to differentiate to Neutrophil-like cells with ATRA (1 µM) for 5 days. The initial rate of CD38 cyclase activity was measured after 1, 3, and 5 days in HL-60 cells (not treated with ATRA) and in differentiated cells. Cyclase activity increased during differentiation (**Figure 1**) confirming the expression of CD38 on the plasma membrane.

Effect of CD38 Expression on NAD levels

To investigate the effect of CD38 expression on NAD levels in cells, NAD levels were determined after 1, 3 and 5 days of differentiation (**Figure 2**).

Data

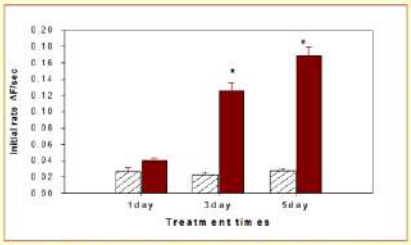

Figure 1: **Time-course of ATRA induced differentiation of HL-60 cells showing the increase in plasma membrane CD38 cyclase activity. (n = 9-11). *P<0.05**

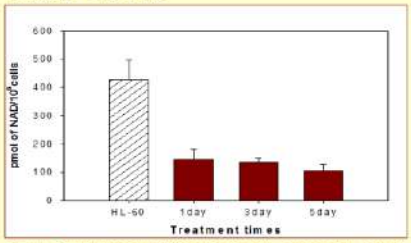

Figure 2: **NAD levels in differentiated cells compared to control (HL-60). Data are mean ± SE, (n=9-11).**

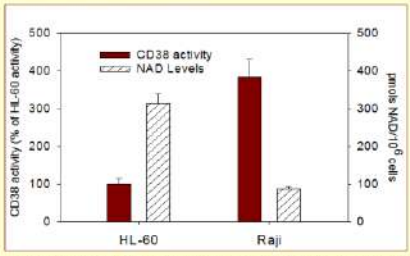

Figure 3: **Cyclase activity (red columns) and NAD levels (hatched columns) in RAJI and HL60 cells. Data are mean ± SE (n = 9-11).**

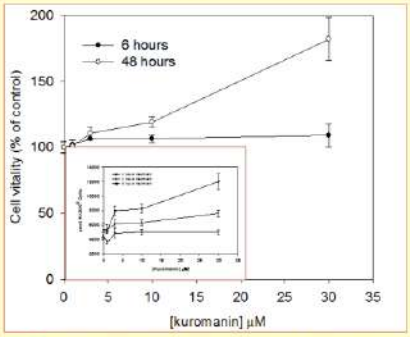

Figure 4: **Effect of kuromanin on Raji cell vitality (MTT assay). Inset, effect of kuromanin on NAD levels in RAJI cells. Data are mean ± SE(n=9)**

Results (cont.)

CD38 and NAD levels in RAJI cells

To confirm that the relationship was not peculiar to HL-60 cells, a CD38+ leukaemic cell line, RAJI was used to confirm our previous results (**Figure 3**). In these cells, expression of high CD38 activity correlated negatively with intracellular NAD levels.

Effect of CD38 inhibition on NAD levels

Inhibition of CD38 was achieved by using the novel flavonoid compound kuromanin. As expected, kuromanin treatment caused a rise in intracellular NAD levels in Raji cells (**Figure 4 inset**).

In order to see the effect of lowered NAD levels on cell vitality, we treated cells with kuromanin and performed the MTT cell vitality assay. The increase in NAD levels was mirrored by an increase in cell vitality.

Discussion

These results would tend to suggest a strict relationship between CD38 expression and NAD levels. We expect that CD38 may be expressed intracellularly, possibly on the nuclear membrane, as well as on the plasma membrane as it is hard to consolidate extracellular enzyme activity with the effect on intracellular NAD levels. In terms of CLL cells expressing CD38, we would expect a similar relationship and thus the reduced NAD levels may play a role in the negative prognosis associated with CD38 expression in patients. However, our initial cell vitality results would tend to suggest that low NAD levels reduce cell vitality.

Future Directions

- Measurement of CD38 expression by PCR/Western Blot in both the Plasma Membrane and intracellular compartments.
- Measurement of intracellular NAD levels in CD38 positive and negative cells from CLL patients.
- Measurement of activity of other NAD consuming enzymes during HL-60 differentiation.
- Further investigation into the physiological consequences of reduced NAD levels in CD38+ cells (eg NAD-dependent metabolism and signaling processes).

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CD38 expression regulates NAD(H) levels during HL-60 differentiation

DISCOVER WITH PLYMOUTH UNIVERSITY

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Background

Human CD38 is a cell surface glycoprotein expressed in a wide variety of cell types that displays both enzymatic and receptorial functions. As a receptor, CD38 controls signaling pathways involved in the activation, growth and survival of lymphoid and myeloid cells. As an enzyme, CD38 uses NAD(P) as a substrate to form a number of biologically active compounds including cADPR, NAADP and ADPR (1) although the major physiological role would seem to be in control of NAD levels. Such control may have wide ranging implications on cell physiology not only due to modulation of basic metabolism but also as NAD has recently been identified as a substrate in a variety of signaling reactions.
CD38 and its metabolites have been proposed to be involved in a number of human diseases ranging from Diabetes to HIV infection. CD38 is also a widely used negative prognostic marker (2) in chronic lymphocytic leukemia (CLL) where increased CD38 expression correlates with a worse prognosis.

Objectives

- To understand the relationship between CD38 expression and NAD levels.
- To understand how this relationship may influence the physiology of CLL cells.

Methods

- HL-60 (CD38-) were treated with all trans retinoic acid (ATRA; 1 μM) to induce differentiation to granulocytes/neutrophil-like cells.
- ADP-ribosyl cyclase activity in whole cells was measured using the fluorescent NGD assay. The initial rate of formation of cGDPR was determined (3). CD38 expression was determined by quantitative real-time PCR using SYBR Green PCR master mix.
- NAD, NADH were extracted as appropriate in acidic/basic conditions and their levels were determined using a modification of the enzymatic cycling assay described by Leonardo et. al. (4).
- Lactate levels were measured using LDH.

Results

CD38 is expressed during differentiation

HL-60 cells were stimulated to differentiate to Neutrophil-like cells with ATRA (1 μM) for 5 days. Cyclase activity was measured after 1, 3, and 5 days. Plasma membrane cyclase activity increased during differentiation (**Figure 1A**) confirming the expression of CD38 on the plasma membrane. **Figure 1B** shows the increase in CD38 mRNA with time during differentiation determined by qPCR.

Effect of CD38 Expression on NAD levels

Intracellular NAD levels were determined after 1, 3 and 5 days of differentiation (**Figure 2**) and were found to be reduced to below 50% of HL-60 levels by 5 days. There was already a significant reduction after one day.

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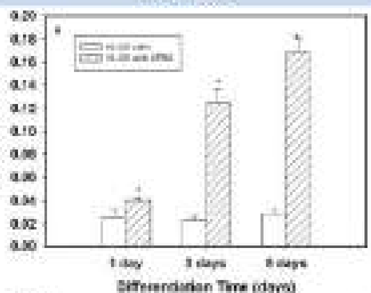
Results



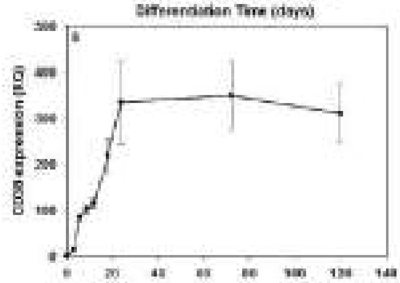


Figure 1: Time-course of ATRA induced differentiation of HL-60 cells showing (a) the increase in plasma membrane CD38 cyclase activity, (n = 6-11), *P<0.05 (b) the increase in CD38 expression, data points represent the means of 8 experiments.

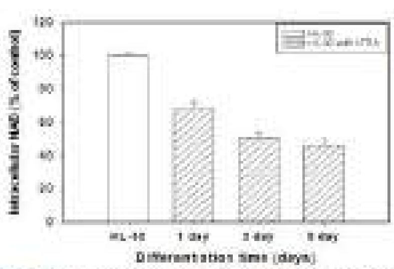


Figure 2: NAD levels in differentiated cells compared to control (HL-60). Data are mean ± SE, (n=6-11).

Effect of CD38 inhibition on NAD levels

Inhibition of CD38 was achieved by using the novel flavonoid compound kuromanin. As expected, kuromanin treatment stopped the reduction in intracellular NAD levels (**Figure 3A**) and seemed to modulate CD38 expression (**Figure 3B**).

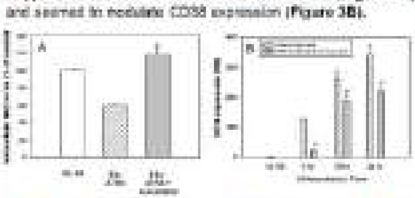


Figure 3: Effect of kuromanin on (A) NAD levels and (B) CD38 expression. Data are mean ± SE (n=6) for 3 experiments.

Results (cont.)

Effect of lowered NAD levels on metabolism

In order to assess the effect of lowered NAD levels on basic cell metabolism, we measured both the NAD+/NADH ratio and (**Figure 4A**) lactate levels (**Figure 4B**). As might be expected, lactate levels were reduced in cells where NAD was depleted probably due to partial inhibition of glycolysis. However, the NAD+/NADH ratio was not significantly altered.

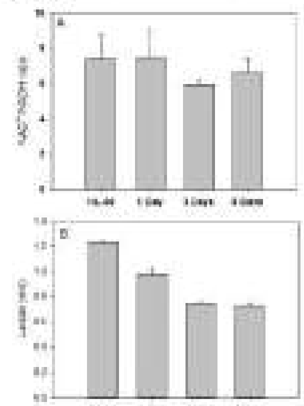


Figure 4: Time-course of ATRA induced differentiation of HL-60 cells showing (a) the NAD/NADH ratio (b) a decrease in lactate levels. Data are mean ± SE, (n=6-11) for 3 experiments.

Discussion

These results would tend to suggest a strict relationship between CD38 expression and NAD levels. We expect that active CD38 may be expressed intracellularly possibly on the nuclear membrane (5,6), as well as on the plasma membrane as it is hard to consolidate extracellular enzyme activity with the effect on intracellular NAD levels.
The results with the novel CD38 inhibitor, kuromanin in CD38 expression studies tend to suggest the exciting possibility that CD38 expression and CD38 activity or NAD levels are linked.
In terms of CLL cells expressing CD38, we would expect a similar relationship and thus the reduced NAD levels may play a role in the negative prognosis associated with CD38 expression in patients although this may not be at the level of basic metabolism.

References

1. Lee HC (2006) Mol Med 12: 317-323.
2. [illegible]
3. Lee HC et. al. (1997) Methods Enzymol 280: 331-340.
4. Leonardo MR, et al. (1996) J Biol Chem [illegible]
5. [illegible]
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RESEARCH WITH PLYMOUTH UNIVERSITY

CD38 Expression Regulates NAD(H) Levels in Human Leukemia Cells

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RESEARCH WITH PLYMOUTH UNIVERSITY

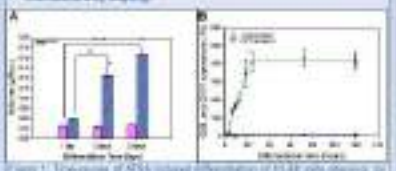
Introduction

CD38 is a multifunctional transmembrane glycoprotein expressed in a wide variety of cell types that displays both enzymatic and receptorial functions. As a receptor, CD38 controls vital cellular functions, such as cell activation, adhesion (binding to CD31), differentiation and survival of lymphoid and myeloid cells. CD38 as an ectoenzyme uses NAD to generate cyclic ADP-ribose (cADPR) and NADP to generate nicotinic acid dinucleotide phosphate (NAADP). These products act as a second messengers that release calcium from intracellular stores (1). Perhaps more importantly, CD38 seems to be able to regulate the levels of extracellular NAD as the wide range of reactions that it performs consume NAD. Such control may have wide ranging implications on cell physiology not only due to metabolism of basic metabolites but also as NAD has recently been identified as a substrate in a variety of signaling reactions. CD38 and its metabolites have been proposed to be involved in a number of human diseases ranging from Diabetes to HIV infection. CD38 is also a widely used as a negative prognostic marker (2) and a disease modifier in chronic lymphocytic leukemia (CLL) where increased CD38 expression correlates with a worse prognosis. While the receptor functions of CD38 are well known little effort has been directed at investigating potential roles of the enzymatic functions in CLL. Here we present data from a CD38-expressing system, HL-60 cells.

Results

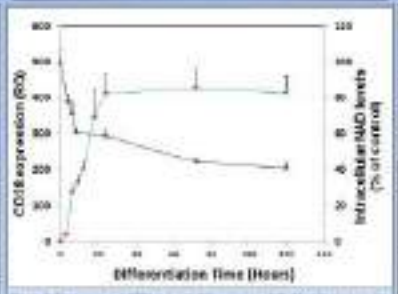
CD38 expression correlates with a drop in intracellular NAD(H) levels

- HL-60 cells were differentiated using 1 μM ATRA for 5 days. Enzymatically active CD38 (measured via the NGD assay) was found on the plasma membrane from 24 hours (Fig 1A).
- CD38 and CD157 (a CD38 homologue) mRNA expression were determined by qPCR (Fig 1B). CD38 expression was upregulated over 400 times during the first 24 hours while CD157 expression increased only slightly.

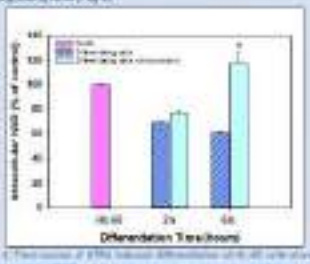


- Western blotting confirmed CD38 protein expression (Fig 2).

- Intracellular NAD levels were determined using an enzymatic assay (Fig 3 black line). Levels fell rapidly with the induction of CD38 expression and by the 5th day of differentiation, were less than 50% of their starting values.



- As the drop in NAD levels seems to follow the increase in CD38 expression closely, we hypothesised that CD38 may be responsible for the change. We used the novel CD38 (enzymatic) inhibitor, Kuromanin, to investigate this relationship. Kuromanin counteracted the drop after 5 hours suggesting that CD38 is degrading NAD (Fig 4).



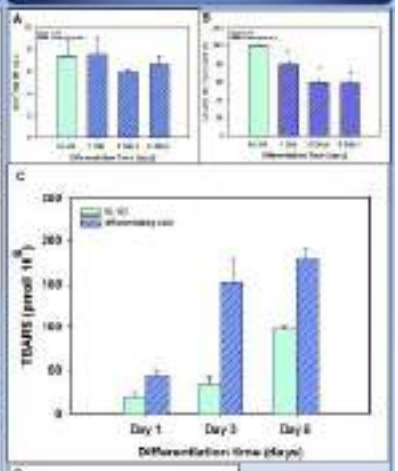
Effect of low intracellular NAD levels on cell physiology

- NAD is an important coenzyme/substrate involved in multiple metabolic and signaling reactions. To assess the effect of lowered NAD levels on basic cell metabolism, we measured both the NAD+/NADH ratio (Fig 5A) and lactate levels (Fig 5B) as glycolytic proxies. As might be expected, glycolytic activity was lowered but the NAD+/NADH ratio was not significantly affected.
- We also measured two indicators of antioxidant capacity, TBARS (Fig 5C) and total glutathione (Fig 5D). A clear increase in TBARS levels (a marker of oxidative stress) was noted.

References

1. Lee HC (2006) Mol Med 12: 317-323.
2. Deaglio S, et al. (2008) Trends Mol Med 14(8): 333-343.

Results



Conclusion

These results suggest a strict relationship between CD38 expression and NAD levels and NAD-dependent processes. In this cell model of CD38 expression. In terms of CLL, cells expressing high levels of CD38 (patients with poor prognosis) would also be expected to show a similar relationship.

It is hard to imagine how a drop in NAD levels associated with increased CD38 expression might be linked to the disease progression in CLL that is associated with increased CD38. We hypothesize that the increase in CD38 expression will be linked with reduced glycolytic activity and an increase in oxidative damage and these characteristics might lead to increased resistance to cell death (as ATP generated via oxidative metabolism is required for apoptosis) and increased cell/DNA damage (presumably via a reduction in activity of a protective mechanism using NADH), possibly leading to increased mutation rates. Furthermore, we are currently investigating whether lower NAD levels might lead to alterations in the DNA damage response via the PARP pathway (that uses NAD for the formation of poly ADP-ribose/PAR). PAR is known as an inducer of apoptosis and lower PARP activity may lead to defective induction of apoptosis and thus cell survival after DNA damage which may lead to accumulation of mutations. These factors may influence the association between CD38 expression and disease progression and prognosis.

The forgotten role of CD38: is enzymatic activity important in CLL pathophysiology?

DISCOVER WITH PLYMOUTH UNIVERSITY

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Background

Human CD38 is a cell surface glycoprotein expressed in a wide variety of cell types that displays both enzymatic and receptorial functions. As a receptor, CD38 controls signaling pathways involved in the activation, growth and survival of lymphoid and myeloid cells. As an enzyme, CD38 uses NAD(P) as a substrate to form a number of biologically active compounds including cADPR, NAADP and ADPR (1) although the major physiological role would seem to be in control of NAD levels. Such control may have wide ranging implications on cell physiology not only due to modulation of basic metabolism but also as NAD has recently been identified as a substrate in a variety of signaling reactions. CD38 and its metabolites have been proposed to be involved in a number of human diseases ranging from Diabetes to HIV infection. CD38 is also a widely used as a negative prognostic marker (2) and a disease modifier in chronic lymphocytic leukemia (CLL) where increased CD38 expression correlates with a worse prognosis. However, as CD38 receptor functions is well known still its enzymatic function need to be illustrated in CLL patients.

Methods

- HL-60 (CD38-) were treated with all trans retinoic acid (ATRA; 1 mM) to induce differentiation to granulocytes/neutrophil-like cells. ADP-ribosyl cyclase activity in whole cells was measured using the fluorescent NGD assay (3). CD38 expression was determined by quantitative real-time PCR using SYBR Green PCR master mix.
- NAD, NADH were extracted as appropriate in acidic/basic conditions and their levels were determined using a modification of the enzymatic cycling assay described by Leonardo et al (4).
- Lactate levels were measured using LDH, and total glutathione was measured as described by Adams et al. (1983) (5).

Results

CD38 is active during differentiation and decreases intracellular NAD levels

HL-60 cells were stimulated to differentiate to Neutrophil-like cells with ATRA (1 μM) for 5 days. CD38 mRNA expression was measured after 3 hours to 5 days. (**Figure 1A red line**) shows the increase with time as determined by qPCR. Intracellular NAD levels were determined after 1, 3 and 5 days of differentiation (**Figure 1A blue line**) and were found to be reduced to below 50% of HL-60 levels by 5 days confirming the effect of CD38 expression on NAD levels. However, inhibition of CD38 by using the novel flavonoid compound (kuromanin) stopped the reduction in intracellular NAD levels (**Figure 1B**).

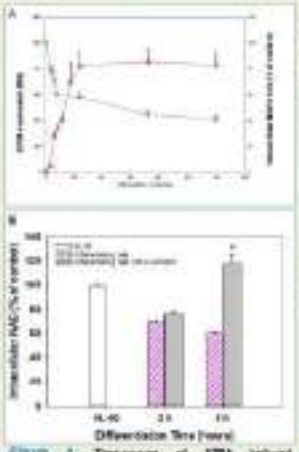

Figure 1: Time-course of ATRA induced differentiation of HL-60 cells showing: (a) The increase in CD38 expression (red line), the decrease in NAD levels (blue line). (b) Effect of kuromanin on NAD levels.

Effect of decrease of extracellular NAD on NAD-dependent processes

In order to assess the effect of lowered NAD levels on basic cell metabolism, we measured both the NAD/NADH ratio (**Figure 2A**) and lactate levels (**Figure 2B**). As might be expected lactate levels were reduced in cells where NAD was depleted probably due to partial inhibition of glycolysis. However, the NAD/NADH ratio was not significantly altered. The antioxidant state represented by total glutathione was also measured (**Figure 2C**) which shows significant increase in GSH levels during the first day. Moreover a clear increase in TBARS levels (a markers of oxidative stress) is shown during days 3 and 5 of differentiation (**Figure 2D**).

It would perhaps seem surprising that a decrease in intracellular NAD(H) levels of c. 60% does not have significant effects on intracellular NAD-dependent processes and perhaps this is symptomatic of how much spare capacity the cell retains for NAD-dependent processes. One final process that may be affected is the activity of PARP enzymes and DNA repair and we are currently investigating this.

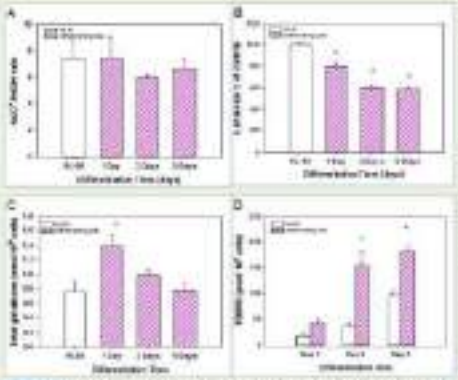

Figure 2: Time-course of ATRA induced differentiation of HL-60 cells showing: (a) The NAD/NADH ratio. (b) A decrease in lactate levels (c) The drop in total glutathione and the increase in (d) Oxidative damage represented by TBARS levels.

Is CD38 expression NAD dependent?

In order to inhibit the NAD recycling pathway we used the NAMPTase inhibitor (FK866) and also we used the extracellular NAD application to increase intracellular NAD. The results show that treatment of HL-60 during the differentiation (**Figure 3A**) for 1 day either with 100 nM of the inhibitor (FK866) and or incubation of cells with 100 μM NAD, resulted in a significant drop in the CD38 expression. Moreover, inhibition of CD38 by kuromanin seemed to modulate CD38 expression (**Figure 3B**).

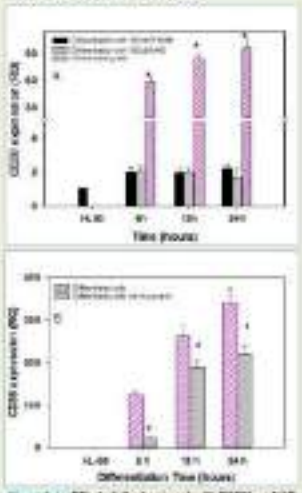

Figure 3: (a) Effect of the treatment with FK866 or NAD on CD38 expression in differentiating cells. (b) Effect of kuromanin on CD38 expression in time course of HL60 differentiation.

Discussion

These results would tend to suggest a strict relationship between CD38 expression and NAD levels. We expect that active CD38 may be expressed intracellularly possibly on the nuclear membrane (5,6), as well as on the plasma membrane as it is hard to consolidate extracellular enzyme activity with the effect on intracellular NAD levels. The results with the novel CD38 inhibitor, kuromanin in CD38 expression studies tend to suggest the exciting possibility that CD38 expression and CD38 activity or NAD levels are linked.

In terms of CLL cells expressing CD38, we would expect a similar relationship and thus the reduced NAD levels may play a role in the negative prognosis associated with CD38 expression in patients although this may not be at the level of basic metabolism.

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Biochemical and Biophysical Research Communications xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Large changes in NAD levels associated with CD38 expression during HL-60 cell differentiation

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ARTICLE INFO

Article history:
Received 29 October 2013
Available online xxxx

Keywords:
NAD(P)
CD38
HL-60
CLL
CD157
Pyridine nucleotides

ABSTRACT

NAD is an important cofactor involved in multiple metabolic reactions and as a substrate for several NAD-dependent signalling enzymes. One such enzyme is CD38 which, alongside synthesising Ca^{2+}-releasing second messengers and acting as a cell surface receptor, has also been suggested to play a key role in NAD^+ homeostasis. CD38 is well known as a negative prognostic marker in B-CLL but the role of its enzymatic activity has not been studied in depth to date. We have exploited the HL-60 cell line as a model of inducible CD38 expression, to investigate CD38-mediated regulation intracellular NAD^+ levels and the consequences of changes in NAD^+ levels on cell physiology. Intracellular NAD^+ levels fell with increasing CD38 expression and this was reversed with the CD38 inhibitor, kuromanin confirming the key role of CD38 in NAD^+ homeostasis. We also measured the consequences of CD38 expression during the differentiation on a number of functions linked to NAD^+ and we show that some but not all NAD^+-dependent processes are significantly affected by the lowered NAD^+ levels. These data suggest that both functional roles of CD38 might be important in the pathogenesis of B-CLL.

1. Introduction

The pyridine nucleotides nicotinamide adenine dinucleotide (NAD) and its phosphorylated form NADP have long been known to be essential co-enzymes in some of the most fundamental reaction pathways of basic metabolism such as glycolysis, the TCA cycle and the pentose phosphate pathway [1]. It has become clear over the past two decades that their roles in cells extend far beyond being simple electron carriers and NAD(P) has also been shown to be a substrate for enzymes that control pathways of DNA repair (via poly ADP-ribose polymerase; PARP), post-translational protein modification (via ADP-ribosyl transferases; ARTs), gene expression (via sirtuins) and Ca^{2+}-signalling (via CD38/CD157; [2–5]). This has led to a renewed interest in the pathways of NAD(P) homeostasis as it is clear that both the oxidation state and absolute levels of NAD(P) will affect cell physiology via a number of pathways. There has also been much interest in these homeostasis pathways as potential pharmacological targets for a wide variety of diseases.

When NAD(P) is used as a substrate rather than as a redox co-enzyme, the result is that the NAD(P) is consumed with all of the reactions above leading to cleavage of the nicotinamide moiety and generation of free nicotinamide along with compounds containing an ADP-ribose (phosphate) group. In order to maintain NAD(P) levels, three distinct pathways exist to re-synthesise NAD(P) [6]. Of the pathways that consume NAD(P), the most important in terms of the general control of NAD(P) levels would appear to be that mediated by the enzyme CD38 as it is apparently constitutively active [7]. Other pathways such as the PARP pathway may also significantly affect intracellular NAD(P) levels under certain conditions (i.e. DNA damage for PARP) but such changes are likely to be transient.

CD38 is an unusual protein in that it possesses both a receptor function, mediating cell–cell contact and proliferation, and an enzymatic activity [8]. Furthermore, the enzymatic activity is unusual in itself in that the enzyme will use multiple pyridine nucleotide substrates and produce multiple products through at least three known enzymatic mechanisms. A number of the enzymatic products of CD38 have been shown to be involved in cell signalling pathways, for instance, cADPR, NAADP and ADPR [9]. While CD38 is clearly an important regulator of the synthesis of second messengers, recent evidence from the CD38 KO mouse has suggested that the principle role of CD38 may be in the control of NAD(P) levels as the KO mouse showed significantly higher tissue NAD levels than the wild-type [10]. CD38 is perhaps best known for being a prognostic marker for chronic lymphocytic leukaemia (CLL) [8]. Briefly, high levels of CD38 expression correlate with both disease stage and poor prognosis. While the receptor functions of CD38 undoubtedly contribute to high levels of cell proliferation in advanced CLL, the contribution of the enzymatic activity in pathogenesis has remained largely ignored.

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.10.170

Please cite this article in press as: Z.N. Al-Abady et al., Large changes in NAD levels associated with CD38 expression during HL-60 cell differentiation, Biochem. Biophys. Res. Commun. (2013), http://dx.doi.org/10.1016/j.bbrc.2013.10.170