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Lactate inhibits germ cell apoptosis in the human testis

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Dysregulation of male germ cell apoptosis has been associated with the pathogenesis of male infertility. Therefore, factors involved in the regulation of germ cell death are being actively investigated. Here, we studied the effects of lactate on human male germ cell death, using as a model a testis tissue culture in which physiological contacts are maintained between the germ cells and the supportive somatic Sertoli cells. Apoptosis of spermatocytes, spermatids and a few spermatogonia was induced by culturing segments of seminiferous tubules under serum-free conditions. This germ cell death was inhibited effectively and dose-dependently by lactate, indicating that it plays a crucial role in controlling cell death cascades of male germ cells. Interestingly, the anti-apoptotic role of lactate was not associated with changes in testicular adenine nucleotide (ATP, ADP and AMP) levels. In the seminiferous tubules, the final site of the death-suppressing action of lactate appeared to be downstream along the cell death pathway activated by the Fas receptor of the germ cells. In conclusion, testicular cell death was effectively regulated by lactate, which may be regarded as a potential compound for optimizing in-vitro methods involving male germ cells for assisted reproduction.

Key words: apoptosis/germ cell/human testis/lactate/spermatogenesis

Introduction

During spermatogenesis, male germ cells proliferate and mature via various phases from spermatogonia through spermatocytes and spermatids to spermatozoa (de Rooij, 1998; Griswold, 1998; de Rooij, 2001). Even during regular conditions, a number of germ cells die by apoptosis before reaching maturity. Thus, normal spermatogenesis involves a balance between cell proliferation and cell death (Dunkel *et al*., 1997a; Rodriguez *et al*., 1997; de Rooij, 2001). In physiological conditions, cell–cell interactions play an important role in controlling this process. Besides giving structural support, the somatic Sertoli cells regulate the fate of germ cells by supplying several paracrine factors (Griswold, 1998). These factors include hormones, several other pro- and anti-apoptotic agents, such as tumour necrosis factor α (TNFα), Fas ligand (FasL) and stem cell factor (SCF), and also energy substrates (Tapanainen *et al*., 1993; Billig *et al*., 1995; Lee *et al*., 1997; Griswold, 1998; Mauduit *et al*., 1999b; Pentikainen *et al*., 1999; Tres and Kierszenbaum, 1999; Francavilla *et al*., 2000; D'Alessio *et al*., 2001; Koji *et al*., 2001; Pentikainen *et al*., 2001; Riera *et al*., 2001; Yan *et al*., 2000a,b,c).

Lactate is one of the compounds produced by the Sertoli cells and utilized primarily by the germ cells. Glucose in the Sertoli cells is metabolized via cytosolic glycolysis to lactate, which is then used by the germ cells as a substrate for ATP production in mitochondrial oxidative phosphorylation (Robinson and Fritz, 1981; Grootegoed *et al*., 1984; Nakamura *et al*., 1984; Bajpai *et al*., 1998; Riera *et al*., 2001). In non-testicular cells, the role of lactate in controlling cell death seems to vary with the specific cell type and the induction pathway of apoptosis. For example, in several cell types, lactate reduces cell growth or further induces cell death triggered by oxidative stress (Cruz *et al*., 2000; Kang *et al*., 2001), whereas in other cell types it has no effect on hypoxia-induced apoptosis (Malhotra and Brosius, 1999). Furthermore, inhibitors of lactate production sensitize certain cells to drug-induced death (Salomon *et al*., 2000). In the regulation of male germ cell apoptosis, the role of lactate has remained unknown.

Interestingly, deprivation of lactate has been shown to decrease the viability of male germ cells (Trejo *et al*., 1995). Moreover, treatment with lactate has been demonstrated to suppress germ cell loss and to improve spermatogenesis in the cryptorchid rat testis *in vivo* (Courtens and Ploen, 1999). Thus, lactate appears to act as a testicular survival factor. The aim of the present study was to evaluate the effects of lactate on human male germ cell death. We used an in-vitro tissue culture model in which the testicular cells remained in the seminiferous

tubules and thus maintained their physiological interactions. To induce apoptosis of human germ cells in this model, we incubated segments of the tubules under serum-free culture conditions, and investigated the ability of lactate to prevent this cell death. Furthermore, we studied the effects of lactate and its metabolite pyruvate on the levels of testicular adenine nucleotides (ATP, ADP and AMP). Finally, we tested whether blockade of oxidative phosphorylation or activation of the Fas receptor would modulate the effects of lactate on male germ cell death.

Materials and methods

Patients

Testis tissue was obtained from 10 adult men aged 69–84 years undergoing orchidectomy as treatment for prostate cancer. The patients had received neither hormonal nor chemotherapeutic medication, nor had they received radiotherapy before the operation, and none of them had suffered from cryptorchidism. The operations were performed between October 1999 and May 2001 at the Department of Urology, Helsinki University Central Hospital (Helsinki, Finland). The Ethics Committees of the Departments of Children and Adolescents and of Urology, University of Helsinki, approved the study protocol.

Tissue culture

Segments of seminiferous tubules were cultured instead of isolated cells in order to maintain the physiological cell–cell interactions of the testicular cells. The testis tissues derived from the operations were immediately microdissected on Petri dishes containing phosphate-buffered saline (PBS) supplemented with human serum albumin (Sigma Chemical Co., St Louis, MO, USA). Segments of seminiferous tubules (3–5 mm in length) were isolated and transferred to culture plates. Samples were incubated in tissue culture medium (Nutrient mixture Ham's F10, containing 1100 mg/l of D-glucose and 110 mg/l of sodium pyruvate; Gibco Europe, Paisley, UK), supplemented with 0.1% human serum albumin (Sigma), and 10 µl/ml gentamicin (Gibco). Incubations were performed at 34°C in a humidified atmosphere containing 5% of CO_2, and on all occasions the pH was neutralized prior to culture.

Exposure to lactate or pyruvate

To evaluate the role of lactate on human testicular apoptosis, neutralized lactic acid (lactic acid; equal amounts of the D- and L-isomers; Sigma) was added to the culture medium at final concentrations of 1, 5 and 10 mmol/l, corresponding to its physiological concentrations in the testicular tissue (Courtens and Ploen, 1999). Furthermore, lactate concentrations of 20 and 25 mmol/l were tested. To assess whether concentrations of pyruvate exceeding the basal concentration would mimic the effects of lactate on cell death, extra sodium pyruvate (Sigma) was added to the culture medium at final concentrations of 5 and 10 mmol/l. On all occasions, the pH was neutralized prior to culture.

Blockade of oxidative phosphorylation

Mitochondrial oxidative phosphorylation was blocked with potassium cyanide (KCN, 5 mmol/l, Sigma) which inhibits cytochrome *c* oxidase (complex IV). This concentration was chosen in order to totally inhibit oxidative phosphorylation. Stock solutions of KCN were prepared in Krebs-Henseleit buffer (115 mmol/l NaCl, 3.6 mmol/l KCl, 1.3 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$, 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, pH 7.2). On all occasions, the pH was neutralized prior to culture.

Activation of the Fas receptors

Agonistic anti-Fas antibody has been shown to activate the apoptotic cascade of testicular germ cells (Tres and Kierszenbaum, 1999). To investigate whether activation of the Fas receptors could modulate the anti-apoptotic effects of lactate, agonistic Anti-Fas antibody (Roche Molecular Biochemicals, Mannheim, Germany) was added to final concentrations of 2 and 5 µg/ml.

Southern blot analysis of apoptotic DNA fragmentation

Testis tissue samples were snap-frozen in liquid nitrogen and stored at –80°C until DNA isolation. DNA was extracted by using the Apoptotic DNA Ladder Kit (Roche) according to the manufacturer's instructions, with some modifications, as recently described (Pentikainen *et al*., 2000). After quantification, DNA samples (1 µg) were 3′-end-labelled with digoxigenin-dideoxy-UTP (Dig-dd-UTP; Roche), using the terminal transferase (TdT; Roche) reaction, subjected to electrophoresis on 2% agarose gels, and blotted onto a nylon membrane. DNA was cross-linked to the membrane by UV irradiation. Dig-dd-UTP 3′-end-labelled DNA on the nylon membrane was detected with an antibody reaction involving alkaline phosphatase (Anti-Digoxigenin-AP; Roche), as recently described (Erkkila *et al*., 1997). The chemiluminescence reaction was performed in CSPD solution (Roche) at room temperature for 5 min, and enhanced at 37°C for 15 min, as described (Erkkila *et al*., 1997). X-ray films were exposed to chemiluminescence, after which the films were scanned with a tabletop scanner (Microtec ScanMaker; Microtec International, Inc., Taiwan) and the digitized information (optical density) was analysed with NIH-Image (1.61) analysis software (National Institutes of Health, Bethesda, MD, USA). Low mol. wt DNA fractions [<1.3 kilobases (kb)] of the 0 h sample were set at 1.0 (100%) and the other settings were compared to this. Thus, the results are expressed in relation to the starting (0 h) point.

In-situ end-labelling (ISEL) of apoptotic DNA

Short segments of seminiferous tubules (1–3 mm in length) were gently squashed under coverslips to produce a monolayer of cells. These squash preparations were fixed as previously described (Erkkila *et al*., 1997), after which the samples were dehydrated and stored at –20°C until they were stained. DNA in-situ 3′-end-labelling was performed as described earlier (Pentikainen *et al*., 2000), with modifications. Briefly, after rehydration and permeabilization in a microwave oven for 5 min in 10 mmol/l citric acid (pH 6.0), the samples were preincubated with terminal transferase reaction buffer (potassium cacodylate 1 mol/l, Tris–HCl 125 mmol/l, BSA 1.25 mg/ml, pH 6.6). The DNA in the samples was 3′-end-labelled with Dig-dd-UTP (Roche) by the terminal transferase (TdT, Roche) reaction for 1 h at 37°C. Antidigoxigenin antibody conjugated to horse-radish peroxidase (Anti-Digoxigenin-POD; Roche) and diaminobenzidine (Sigma) were used to detect the Dig-dd-UTP-labelled DNA. The slides were weakly counterstained with haematoxylin, after which the samples were dehydrated. For the negative controls, the TdT enzyme was replaced by the same volume of distilled water.

Electron microscopy

Segments of seminiferous tubules were cultured as described above. They were fixed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer, pH 7.2, postfixed with 1% osmium tetroxide in 0.1 mol/l phosphate buffer, dehydrated, and embedded in epoxy resin. They were then sectioned at 50 nm with a Reichert E ultramicrotome (Reichert Jung, Vienna, Austria) and stained with uranyl acetate and lead citrate. Observations were made with a Jeol JEM 1200 EX transmission electron microscope (Jeol, Tokyo, Japan). Classification of germ cell

types was based on their characteristic morphology and the cells were identified as apoptotic by the changes in their ultrastructure.

Determination of ATP, ADP and AMP

Samples of testicular tissue were snap-frozen in liquid nitrogen. To extract adenine nucleotides (ATP, ADP and AMP), the tissues were homogenized in 0.42 N ice-cold perchloric acid. The homogenates were then neutralized with 4.42 N KOH and centrifuged. The ATP, ADP and AMP concentrations of the supernatants were determined with high performance liquid chromatography (HPLC) using a Shimadzu LC 10AD vp liquid chromatograph with a reversed phase column (Ultra Techsphere 5 ODS; Labtronic Oy, Vantaa, Finland) and an UV detector set at 254 nm. The published method (Stocchi *et al*., 1987) was modified as follows: buffer A (0.1 mol/l KH_2PO_4, 8.0 mmol/l tetrabutylammonium hydrogen sulphate, pH 6.0) was run at 1.5 ml/min for 2.5 min followed by a linear increase during 10 min to 100% buffer B (buffer A with 30% methanol), which was continued for 2.5 min and followed by a linear increase during 1 min to 100% buffer A, which was run for 4 min. The compounds were identified and quantified by the retention times and peak areas of known standards, calibrated by spectrophotometry. The adenine nucleotide concentrations were expressed in relation to wet testis tissue weight (µmol/l/mg of testis). The adenylate energy charges (EC) were calculated from the ATP, ADP and AMP concentrations, according to the following equation:

$$([ATP]+1/2[ADP]) / ([ATP]+[ADP]+([AMP]).$$

Statistical analysis

The experiments were repeated on two to seven independent occasions. For statistical comparisons, data obtained from the replicate experiments (mean ± SEM) were analysed by one-way analysis of variance followed by the post-hoc test with Bonferroni correction. $P < 0.05$ was considered significant.

Results

In-vitro induction of human testicular apoptosis and its suppression by lactate

Male germ cell apoptosis was induced by culturing segments of human seminiferous tubules under serum-free conditions. Incubation for 4 and 24 h resulted in clear apoptotic DNA laddering (Figure 1). During culture for 4 h, this low mol. wt DNA fragmentation was suppressed dose-dependently by lactate. Apoptosis was inhibited by 70% ($P < 0.01$) at a lactate concentration of 10 mmol/l, by 20% (not significant) at 5 mmol/l, and by 14% (not significant) at 1 mmol/l, relative to the 4 h control sample (Figure 1). Apoptosis was also effectively blocked by lactate concentrations exceeding the physiological concentrations, i.e. by 72% at 20 mmol/l and by 73% at 25 mmol/l (data not shown). After incubation for 24 h of culture the anti-apoptotic effect of lactate had disappeared. Since lactate, prior to utilization in mitochondrial oxidative phosphorylation, can be oxidized to pyruvate, we also tested whether pyruvate at concentrations higher than the basal concentration in the culture medium could mimic the anti-apoptotic effects of lactate. At concentrations of 5 mmol/l and 10 mmol/l, pyruvate was unable to inhibit human testicular apoptosis (Figure 1).

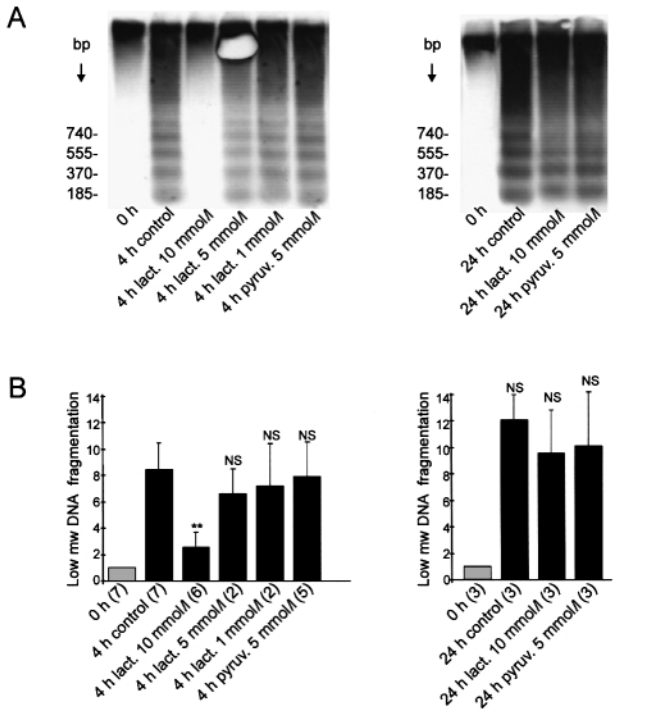

Figure 1. In-vitro induction of human testicular apoptosis and its inhibition by lactate. Segments of human seminiferous tubules were cultured for 4 or 24 h under serum-free culture conditions in the absence or presence of lactate (lact.) or extra pyruvate (pyruv.). (**A**) Southern blot analysis of apoptotic DNA fragmentation. Extracted DNA was 3′-end-labelled with Dig-dd-UTP, subjected to electrophoresis and detected with chemiluminescence. At the start (0 h), no apoptotic fragmentation was observed, whereas incubation for 4 h (4 h control) and 24 h (24 h control) without survival factors induced time-dependent increases in apoptotic laddering. In the 4 h culture, lactate suppressed this testicular apoptosis dose-dependently, but with incubation for 24 h this inhibitory action was no longer observed. Extra pyruvate did not mimic the effects of lactate. (**B**) Quantification of low mol. wt (mw) DNA (<1.3 kb). Each value represents a mean of replicative experiments ± SEM. $^{**}P < 0.01$. The numbers in brackets indicate the numbers of replicative experiments in each treatment. NS = not significant.

Identification of apoptotic cells by in situ 3′-end labelling of DNA (ISEL)

To confirm the results of the Southern blot analyses and to identify the dying cells, we performed ISEL of seminiferous tubule samples (Figure 2). Representative samples of cells from human seminiferous tubules were obtained by squashing segments of seminiferous tubules under coverslips. This method enables the cells from the seminiferous epithelium to move out from the tubules and produce a monolayer on a microscope slide (Figure 2A, small insert). Consistent with the results of Southern blot analyses, induction of germ cell death and its inhibition by lactate, but not by extra pyruvate, was observed in ISEL (Figure 2A). The apoptotic cells were mostly identified as spermatocytes or spermatids (Figure 2B), which is in agreement with our previous studies (Erkkila *et al*., 1997, 1998, 1999). As a new finding, a few human spermatogonia undergoing apoptosis were also observed (Figure 2B). However, many of the spermatogonia were ISEL-negative and their morphology was normal. Because of nuclear pycnosis in the late stages of apoptosis, we could not identify

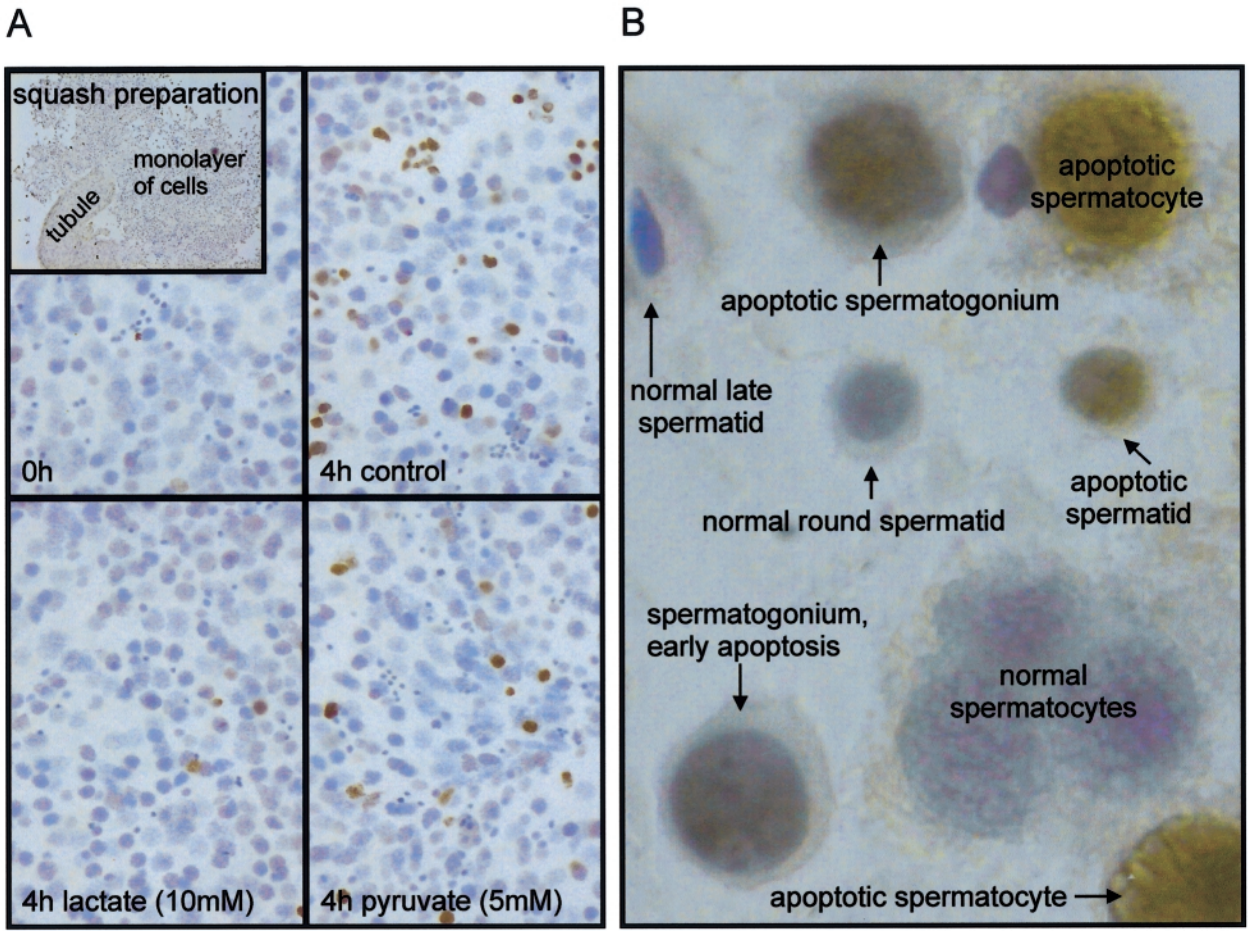

Figure 2. In-situ 3′-end labelling of apoptotic DNA (ISEL). Segments of human seminiferous tubules were cultured, squashed and fixed as described in Materials and methods. Apoptotic cells (brown) were detected by ISEL. (**A**) Small insert demonstrating the squash preparation. Consistent with the results of Southern blot analysis, incubation of testicular tissue under serum-free conditions (4 h control) induced germ cell death which was inhibited by lactate, but not by extra pyruvate. (**B**) The dying cells were identified as mainly spermatocytes and spermatids; however, some spermatogonia undergoing apoptosis were also observed.

all the apoptotic cells. When the terminal transferase enzyme was replaced by the same volume of distilled water as a negative control, there was no staining.

Morphological changes in dying germ cells under the electron microscope

Dying germ cells were further identified by electron microscopy. As in the ISEL analysis, different phases of apoptotic changes, including nuclear and/or cytoplasmic condensation, were observed in spermatocytes (Figure 3) and spermatids. The apoptotic nature of the dying spermatogonia was also confirmed by electron microscopy. In early apoptosis of spermatogonia, clumping of chromatin in the nuclei of the cells was observed (Figure 3). In the later phases of apoptosis, both chromatin and cytoplasm condensed further and the cytoplasmic organelles degenerated (Figure 3). As in ISEL, in very late phases of cell death the specific cell types could not be identified.

The death-suppressing role of lactate is not modulated by concomitant blockade of oxidative phosphorylation

Since one of the main functions of lactate is participation in cellular energy metabolism, we evaluated whether manipulation of energy production could modify the anti-apoptotic effect of lactate. Because lactate is consumed by oxidative phosphorylation in germ cells (Nakamura *et al*., 1984; Bajpai *et al*., 1998), we tested whether blockade of oxidative phosphorylation would affect the anti-apoptotic role of lactate. As shown by Southern blot analysis of DNA fragmentation, potassium cyanide (KCN, 5 mmol/l), which inhibits oxidative phosphorylation, did not modulate the death-suppressing role of lactate (Figure 4).

The anti-apoptotic effect of lactate is not related to changes in adenine nucleotide levels

Because adenine nucleotides (ATP, ADP and AMP) or their relative concentrations have been shown to play an important role in regulating cell death (Catisti *et al*., 1999; Leist *et al*., 1999; Vander Heiden *et al*., 1999; Bradbury *et al*., 2000; Single *et al*., 2001), we studied whether lactate affects the testicular adenine nucleotide levels. The ANs were measured after culture for 4 h, since it is the time point of effective suppression of testicular apoptosis by lactate. No significant differences in ATP, ADP or AMP concentrations (Figure 5) or ATP/ADP proportions or adenylate energy charges (data not shown) were observed between the samples treated with lactate

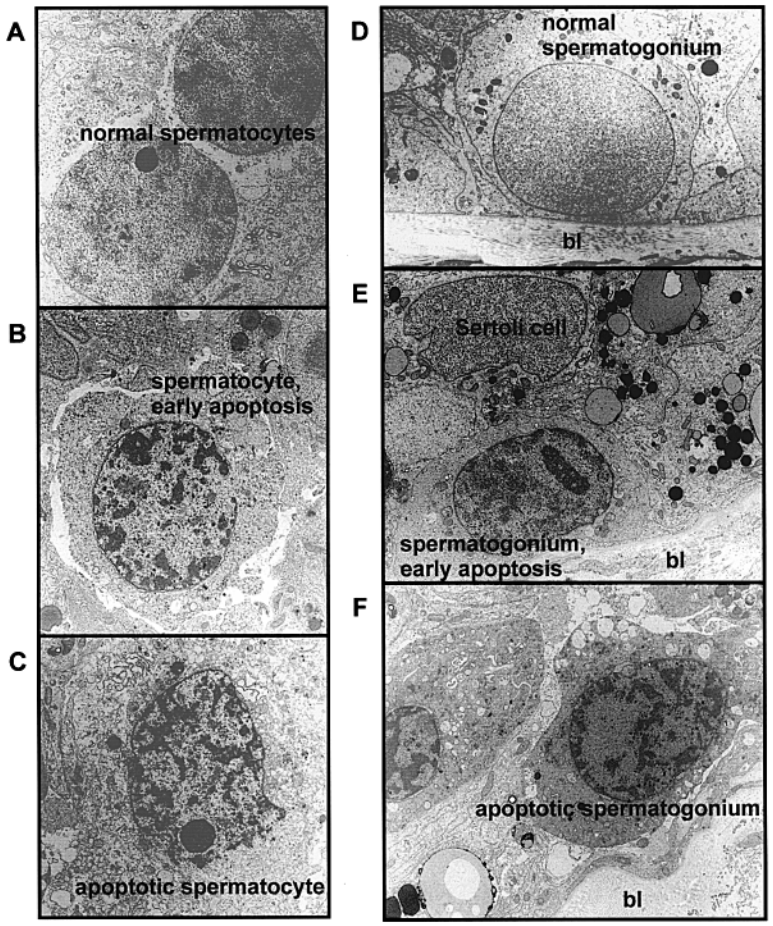

Figure 3. Electron micrographs of cells from the human testis. Segments of seminiferous tubules were cultured under serum-free conditions to induce germ cell apoptosis. (**A**–C) Different stages of apoptosis in spermatocytes. (**A**) Normal spermatocytes. (**B**) Early apoptosis of a spermatocyte. The chromatin has condensed and condensation of the cytoplasm has also begun. (**C**) At a later stage of apoptosis, condensation of the chromatin is more advanced, and the cytoplasmic organelles are no longer identifiable. (**D**–**F**) Apoptosis of human spermatogonia. (**D**) A normal spermatogonium, which is characteristically located next to the basal lamina (bl). (**E**) Early apoptosis of a spermatogonium. Clumping of chromatin in the nucleus as well as condensation of cytoplasm have begun. The upper nucleus belongs to a normal non-apoptotic Sertoli cell. (**F**) In a later phase of apoptosis in a spermatogonium, both the chromatin and the cytoplasm have condensed further and the cytoplasmic organelles have degenerated.

and the control samples and the samples treated with extra pyruvate. Therefore, the anti-apoptotic effect of lactate appears to be mediated by a mechanism that is not explained by changes in the adenine nucleotide levels.

Agonistic anti-Fas antibody does not induce apoptosis in the presence of lactate

The Fas system is known to play a proapoptotic role in our tissue culture model (Pentikainen *et al*., 1999). In order to evaluate whether the anti-apoptotic effect of lactate takes place upstream or downstream of the Fas receptor activation in the germ cells, we added an agonistic anti-Fas antibody to the cultures. In Southern blot analysis of DNA fragmentation, the anti-apoptotic role of lactate was not modified by the activating anti-Fas antibody (Figure 6), suggesting that lactate acts in germ cells downstream of the Fas receptor. The agonistic anti-Fas antibody by itself did not significantly induce further apoptotic germ cell death, which was triggered by serum withdrawal. This was expected, since we have previously shown that, in cultured human seminiferous tubules, the Fas system is activated endogenously in serum-free conditions and, therefore, may have already reached its maximal rate of activation (Pentikainen *et al*., 1999).

Discussion

In the present study, physiological (and higher) concentrations of lactate were shown to effectively suppress human male germ cell apoptosis. Culturing segments of seminiferous tubules under serum-free conditions induced germ cell apoptosis, which, after 4 h of incubation, was inhibited by lactate. Interestingly, blockade of oxidative phosphorylation did not modify the anti-apoptotic effect of lactate. Furthermore, the death-suppressing effect of lactate was not associated with changes in adenine nucleotide (ATP, ADP or AMP) levels. Finally, activation of proapoptotic Fas receptors in the presence of lactate did not induce apoptosis, suggesting that the anti-apoptotic action of lactate takes place downstream along the cell death pathway activated by the Fas receptor.

Consistently with our previous studies (Erkkila *et al*., 1997,

1998, 1999), the germ cells in the later phases of differentiation were the most sensitive to death-inducing conditions, since the apoptotic cells were primarily identified as spermatocytes and spermatids. However, as a new finding, human spermatogonia undergoing apoptotic changes were also characterized. Our failure to observe apoptosis in spermatogonia in earlier studies may have either been due to difficulties in identification of some of the cells because of cellular pycnosis, or to the relatively small number of spermatogonia of which only few appear to undergo apoptosis in the testis of adult men. In rodents, spermatogonial apoptosis not only takes place when normal spermatogenesis is disturbed, but is also part of a necessary pathway to regulate the number of maturing germ cells (de Rooij, 1998; Tres and Kierszenbaum, 1999; Meng *et al*., 2000; Yan *et al*., 2000a,b,c; de Rooij, 2001; Sugiyama *et al*., 2001). In humans, spermatogonial apoptosis has been described in the testes of adult men and cryptorchid boys (Heiskanen *et al*., 1996; Dunkel *et al*., 1997b; Oldereid *et al*., 2001). Our finding that some spermatogonia undergo increased apoptosis under stress-inducing conditions is consistent with these previous reports. However, the role of spermatogonial death in testicular physiology and pathology in humans and other primates is not yet clear and remains to be further clarified.

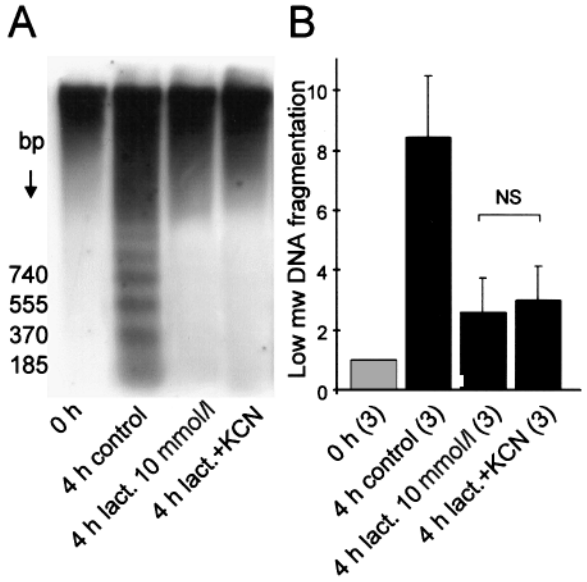

Figure 4. The anti-apoptotic effect of lactate (lact.) is not modulated by concomitant blockade of oxidative phosphorylation. Human testicular tissue was cultured under serum-free culture conditions for 4 h in the absence or presence of lactate and KCN (5 mmol/l) which inhibits oxidative phosphorylation. (**A**) Southern blot analysis of DNA fragmentation. The death-suppressing role of lactate was not modified by concomitant blockade of oxidative phosphorylation. (**B**) Quantification of low mol. wt (mw) DNA (<1.3 kb) fragmentation. Each value represents a mean of replicative experiments ± SEM. The numbers in brackets indicate the numbers of replicative experiments in each treatment.

Human germ cell death was effectively inhibited with lactate after 4 h of culture. In rats, a recent study demonstrated that intratesticular infusion of lactate improves spermatogenesis by preventing loss of germ cells in the cryptorchid testis (Courtens and Ploen, 1999). However, in the present as well as in the rat study, loss of germ cells continued after a delay, despite the lactate treatment. In the rat study, the delay lasted for days, whereas, in our study, clear DNA laddering indicating cell death was observed after 24 h. These different time courses may either reflect species-specific differences in the sensitivity of the germ cells to apoptosis, or result from different death-inducing stimuli. Nevertheless, both these studies clearly suggest that lactate plays an important role in the control of germ cell death. This is further supported by the finding that pharmacological deprivation of lactate decreases the viability of male germ cells (Trejo *et al*., 1995). Since lactate is a normal physiological metabolite, it lacks many of the potential side-effects of non-physiological factors. It can therefore be regarded as a potential therapeutic compound when optimizing treatments for male infertility. Such situations may include attempts to maintain the viability of the testicular tissue *in vitro*, when seeking spermatids or spermatozoa from testicular biopsies for fertilizations.

Lactate in germ cells has been suggested to be consumed by mitochondrial oxidative phosphorylation (Robinson and Fritz, 1981; Nakamura *et al*., 1984). However, the suppressive role of lactate on testicular apoptosis seems not to be mediated

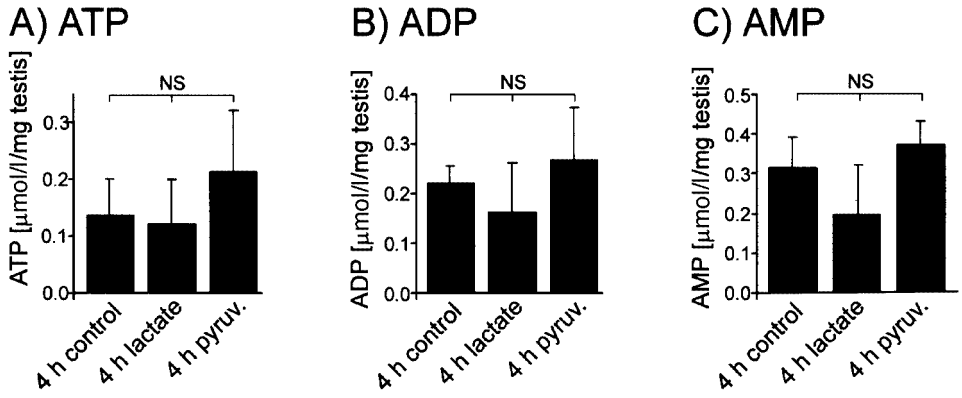

Figure 5. High-performance liquid chromatography (HPLC) analysis of adenine nucleotides. Segments of seminiferous tubules were cultured under serum-free conditions in culture medium that contained a basal level of pyruvate (pyruv.), in the absence or presence of lactate (10 mmol/l) or extra pyruvate (5 mmol/l). HPLC analysis of adenine nucleotides (ATP, ADP and AMP) was performed as described in Materials and methods. No significant differences in ATP, ADP or AMP concentrations were observed between the control samples and the samples treated with lactate or extra pyruvate. Each value represents a mean of three replicative experiments ± SEM.

via the ATP production in oxidative phosphorylation, since lactate inhibited cell death even after blocking oxidative phosphorylation. This is further supported by the finding that ATP levels in the lactate-treated samples did not significantly differ from that in the other samples. Moreover, the anti-apoptotic effect of lactate did not appear to be mediated by changes in the levels of other adenine nucleotides (ADP, AMP) or by the relative concentrations of ATP and ADP (ATP/ADP) or the adenylate energy charges. However, we measured the adenine nucleotide levels after culture for 4 h, and the possibility that lactate could have altered the adenine nucleotide levels at some other time point cannot be excluded. Since the adenine nucleotides of specific cell types were not measured, changes in individual cells cannot be ruled out either. The absence of differences between the ATP levels of the controls and the other samples may be explained by the fact that the culture medium contained glucose and pyruvate. Pyruvate can be utilized to produce ATP via oxidative phosphorylation in the germ cells (Grootegoed *et al*., 1984; Nakamura *et al*., 1984; Bajpai *et al*., 1998). Glucose can also be metabolized to ATP by the Sertoli cells, and possibly also by the germ cells (Robinson and Fritz, 1981; Grootegoed *et al*., 1984; Nakamura *et al*., 1984; Bajpai *et al*., 1998). Moreover, the glucose in the Sertoli cells could have been metabolized to lactate or pyruvate, which could then have been consumed in oxidative phosphorylation of the germ cells. However, if this hypothetical endogenous lactate production occurs in the present culture model, it is likely to be sufficient for maintaining the ATP levels but not for causing the anti-apoptotic effects of lactate.

The mechanism underlying the role of lactate in preventing germ cell death is unclear. The previously reported anti-apoptotic effects of pyruvate, a metabolite of lactate, have been suggested to be mediated via its anti-oxidant capacity or via products of its metabolism in the mitochondrial matrix compartment (Miwa *et al*., 2000; Kang *et al*., 2001). Since, in the present study, even high concentrations of pyruvate had no effects on testicular cell death, it is unlikely that these mechanisms played a role in the present findings. Furthermore, we showed that inhibition of testicular cell death by lactate appeared not to be explained by the changes in adenine nucleotide levels. However, lactate has several other targets of action that may be related to its death-suppressing effects. For example, lactate has been associated with the actions of stem cell factor (SCF), tumour necrosis factor α and FSH (Mauduit *et al*., 1999a; Grataroli *et al*., 2000; Hahn *et al*., 2000; Riera *et al*., 2001), which, in turn, have been suggested to be testicular anti-apoptotic factors (Tapanainen *et al*., 1993; Shetty *et al*., 1996; Mauduit *et al*., 1999b; Pentikainen *et al*., 2001; Tesarik *et al*., 2000; Yan *et al*., 2000a,b,c). Moreover, lactate may have altered the pH levels during the incubations, even though the pH was adjusted prior to the culture. Changes in pH levels may affect testicular apoptosis, firstly, since expression of SCF, a germ cell survival factor, in Sertoli cells depends on pH (Mauduit *et al*., 1999a), and secondly, since at least in several non-testicular cells, pH levels play a crucial role in cell death events (Khaled *et al*., 2001). However, among these and other possible mechanisms, the one(s) explaining the anti-apoptotic role of lactate remain to be further clarified.

Since cell–cell interactions play an important role in germ cell survival, it is possible that lactate acts on the Sertoli cells rather than the germ cells by modulating the supply of pro- or anti-apoptotic paracrine factors. The present *in-vitro* model, in which we cultured segments of seminiferous tubules, has the advantage of maintaining physiological contacts between the different cell types, and thus allows investigation of paracrine systems. One such system, which has been shown to regulate germ cell death in the testis and also in our *in-vitro* model, is the Fas-Fas ligand (FasL) system (Lee *et al*., 1997; Pentikainen *et al*., 1999; Tres and Kierszenbaum, 1999; Francavilla *et al*., 2000; D'Alessio *et al*., 2001; Koji *et al*., 2001). The proapoptotic FasL expressed by the Sertoli cells,

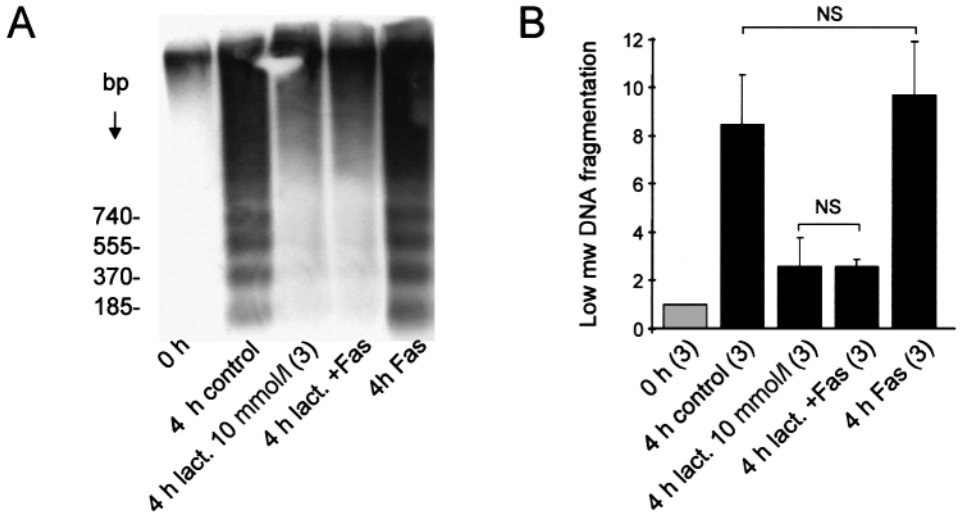

Figure 6. Activating anti-Fas antibody does not induce apoptosis in the presence of lactate. Segments of seminiferous tubules were cultured under serum-free culture conditions in the absence or presence of lactate (10 mmol/l) and/or agonistic anti-Fas antibody (5 μg/ml). (**A**) Southern blot analysis of DNA fragmentation. Activation of the Fas receptor by agonistic anti-Fas antibody did not modulate the death-suppressive effect of lactate. (**B**) Quantification of low molecular weight DNA (<1.3 kb) fragmentation. Each value represents a mean of three replicative experiments ± SEM.

and perhaps also by the germ cells (D'Alessio *et al*., 2001), activates the Fas receptors, and thereafter the apoptotic cascade in the germ cells (Lee *et al*., 1997; Pentikainen *et al*., 1999; Tres and Kierszenbaum, 1999; Francavilla *et al*., 2000; Koji *et al*., 2001). To evaluate the level at which lactate exerts its anti-apoptotic effect, we added Fas receptor-activating antibodies to the cultures. In the presence of lactate, activation of the Fas receptors did not induce apoptosis, which suggests firstly, that lactate may inhibit the particular apoptotic pathway which is triggered by Fas, and secondly, that the inhibitory action of lactate seems to take place downstream of the Fas receptors in the germ cells. However, it is also possible that lactate acts on the Sertoli cells, inducing in them activation of some anti-apoptotic factor(s) which, in turn, act(s) on germ cells downstream of the Fas receptors.

In conclusion, the present study suggests an important role for lactate in the regulation of human male germ cell death. Apoptosis of spermatocytes, spermatids and a few spermatogonia was induced by culturing segments of seminiferous tubules under serum-free conditions. This cell death was effectively inhibited by lactate, which thus appears to have a crucial role in controlling cell death cascades of male germ cells. The anti-apoptotic effect of lactate was not associated with changes in adenine nucleotide levels. In the seminiferous tubules, which contain several interacting cells, the final site of the death-suppressing action of lactate appeared to take place in germ cells downstream of the Fas receptor activation. The results obtained in the present study could be used when optimizing in-vitro methods involving male germ cells for assisted reproduction.

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