The use of human umbilical cord blood serum is beneficial for the continuous production of hepatitis C virus

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In this study we investigated if human umbilical cord blood serum (CBS) is a suitable replacement for foetal bovine serum (FBS) in cultures of human hepatoma cell line Huh7.5, particularly regarding its capacity to maintain high growth rates, differentiation status and its ability to support robust hepatitis C virus (HCV) infection. Generally, CBS-cultured Huh7.5 cells remained comparable to FBS-cultured cells, and proliferated equally well. Albumin secretion, a hepatocyte differentiation marker, had increased 8x in CBS; however, most other hepatocyte markers we tested had not changed. Surprisingly, CBS-cultured cells were able to sustain very high levels of HCV production, and HCV infection in CBS-cultured cells did not induce cell lysis, which is typically seen in HCV-infected cells cultured in FBS. We discuss some of the differences between CBS, adult human serum and FBS that may explain the differences observed.

Hepatitis C virus (HCV) chronically infects an estimated 130–170 million people worldwide. The infection increases the risk of developing liver cirrhosis and liver cancer, and results in more than 350 000 deaths annually. Presently, the main HCV culture system uses human hepatoma Huh-7 or Huh7.5 cells, cultured in media containing FBS (foetal bovine serum), that are transfected or infected with a full-length HCV genome (JFH-1). This culture system supports robust levels of viral production (Lindenbach et al., 2006; Wakita et al., 2005; Zhong et al., 2005).

Recently, we showed that when we cultured Huh-7 or Huh7.5 cells in media containing adult human serum (HS), growth was arrested and they differentiated into cells that functionally and morphologically resembled primary hepatocytes (Steenbergen et al., 2013). This also resulted in a 1000-fold increase in HCV titres when cells where infected with WT JFH-1. However, the observed growth arrest may be detrimental for some applications. For example, in the case of HS-cultured Huh7.5 cells, initial HCV infection rates of growth-arrested HS-cultured cells are low compared with FBS-cultured cells, possibly because these cells are, by necessity, grown to confluency. The use of FBS or other xenogeneic animal sera may also be undesirable, for example due to the potential contamination with bovine prions or other pathogens. To address this concern, serum-free cultures have been developed, as well as cell culture media with alternative growth supplements (Mallon et al., 2006), such as human amniotic fluid or human umbilical cord blood serum (CBS). Human umbilical cord blood is the blood left in the umbilical cord after the birth of an infant. The umbilical cord would usually be discarded as medical waste after delivery. The use of CBS as a replacement for FBS has been described in primary islet cell cultures (Xia et al., 2004) and mesenchyme-like cells (Shetty et al., 2007). In addition, the growth-promoting properties of CBS have been demonstrated in some clinical applications (Ang et al., 2011; Navone et al., 2013; Vajpayee et al., 2003; Yoon et al., 2007a, b).

In addition to our previous study comparing FBS, ABS (adult bovine serum) and (adult) HS, here we present a brief assessment of the effects of CBS on Huh7.5 cells. In particular, we investigated if CBS is a suitable replacement for FBS, with regards to its capacity to maintain high growth rates, and its ability to induce differentiation and support robust HCV infection. Umbilical cords were obtained from healthy voluntary donors who had given informed consent. CBS was isolated, pooled and heat inactivated at 56 °C for 30 min before use. To test if CBS had a similar proliferative capacity as FBS, cells were plated on media containing either FBS or CBS (DMEM, 10% FBS or CBS, penicillin, streptomycin) and cell numbers were determined for 30 days (5 passages) using an automated cell counter. At each passage, 100 000 cells were replated and cells were counted at days 2 to 7 post plating. Cells cultured...
in CBS medium had similar or marginally higher proliferation rates compared with cells in FBS medium (Fig. 1a). We did not observe any morphological changes, and no increased cell death, when observing the cell cultures under a microscope, even after prolonged maintenance of cells in CBS media.

We then determined how CBS cells compared with FBS- and HS-cultured cells with regards to a panel of mRNAs that were shown to be important in the life cycle of HCV. Because of its function in both tight junction formation and in the entry of HCV, we first analysed, by quantitative PCR (Steenbergen et al., 2013), if culturing in CBS resulted in changes in the mRNA levels of tight junction components claudin-1 and occludin. Consistent with the proliferative character of CBS-cultured cells, there was no significant increase in claudin-1 or occludin mRNA (Fig. 1b, c). Additionally, we compared mRNA levels of three HCV entry receptors (CD81, SR-b1, EGFr) in CBS- and FBS-cultured cells, and found no significant changes, except for SR-b1, which was increased approximately 1.5-fold (Fig. 1d–f).

We also wanted to determine if some hepatocyte-specific processes were restored in CBS. We have shown previously that albumin and very-low-density lipoprotein (VLDL) secretion was increased or restored in Huh7.5 cells that were cultured in HS, while these functions were suppressed or absent in cells cultured in FBS medium (Steenbergen et al., 2013). Methods to determine VLDL secretion have been described previously (Steenbergen et al., 2013). Unlike cells grown in HS, cells

![Fig. 1. Characterization of FBS- and CBS-cultured Huh7.5 cells. (a) Cell proliferation in FBS or CBS was assessed on an automated cell counter for 30 days. Cells in FBS and CBS proliferated at approximately the same rate. Open circles: CBS-cultured Huh7.5 cells. Closed circles: FBS-cultured Huh7.5 cells. (b–f) mRNA levels of tight junction proteins and HCV entry receptors were determined by quantitative PCR. (b, c) mRNA levels of tight junction components claudin-1 and occludin were not significantly changed in CBS, but were increased in HS. (d–f) No significant changes were measured in mRNAs of CD81 and EGFr; mRNA levels of SR-b1 increased approximately 50% in CBS and HS (P<0.05). Data are expressed as means with standard deviations (n=3); asterisks indicate significant changes, P<0.05.](http://jgv.microbiologyresearch.org)
cultured in CBS did not secrete VLDL, even after prolonged culture in CBS (Fig. 2a): CBS cells, like FBS-cultured cells, do secrete low-density lipoprotein (LDL)-sized lipoprotein particles, but the VLDL-sized peak that is observed in HS (after 21–30 days) was not observed in cells cultured in media containing either FBS or CBS.

Secretion of albumin, and other serum proteins, is an important function of hepatocytes. Albumin is the most abundant protein in human serum, constituting approximately half of all serum protein. Albumin secretion is therefore often used as a marker of hepatocyte differentiation or hepatocyte functionality (Ayatollahi et al., 2011; Sainz & Chisari, 2006). We measured the quantities of albumin secreted by Huh7.5 cells that were cultured in media containing FBS, ABS (adult bovine serum), CBS or HS, as described previously (Steenbergen et al., 2013). After approximately 1 week in CBS, albumin secretion increased approximately eightfold, compared with that in FBS-cultured cells, and these levels were maintained for at least 8 weeks after the transfer of cells to CBS-containing medium (Fig. 2b). The amount of albumin secretion in CBS-containing cultures was comparable to the amount of albumin secreted by fully differentiated HS-cultured Huh7.5 cells. ABS can be seen as the bovine counterpart of HS, as both are low in growth factors. Like culturing in HS, culturing in ABS results in growth arrest of Huh7.5 cells, presumably due to the lack of growth factors. Albumin secretion, however, remained low in ABS-cultured cells, and comparable to that in FBS-cultured cells.

The increase in albumin secretion in CBS-cultured cells indicates a higher degree of hepatocyte functionality, compared with their FBS and ABS counterparts, but the lack of VLDL secretion indicates incomplete differentiation, as might be expected from rapidly dividing cells. The lack of VLDL secretion in CBS-cultured cells may in fact be explained by their proliferative state and its associated metabolic profile. In contrast to non-dividing cells, which rely primarily on mitochondrial oxidative phosphorylation to generate energy, most dividing cells rely on aerobic glycolysis (Vander Heiden et al., 2009). Aerobic glycolysis is an inefficient way to produce ATP; however, the benefit to the dividing cells may be that it facilitates the conversion of nutrients (pyruvate) into biomass (e.g. nucleotides, amino acids and lipids), which is needed to produce new cells. We have shown that growth-arrested cells in adult HS undergo complete metabolic programming, away from this proliferative metabolic profile. HS-cultured cells also have much larger glycogen and lipid stores than dividing cells in FBS (unpublished data). The availability of large lipid stores might in turn enable processes that depend on the availability of excess lipids inside the cell, like VLDL secretion.

In the last part of this study, we determined the ability of viral production by Huh7.5 cells cultured in FBS and CBS. Cells were infected with a tissue-culture-adapted variant of JFH-1, which produces high viral titres in FBS-cultured cells. We first determined the presence and distribution of HCV core in infected cultures as described previously (Steenbergen et al., 2013). As early as 3 days post-infection, more cells were HCV-positive in CBS media compared with FBS media, indicating higher initial infection rates, faster replication or faster spread. On day 3 post-infection, approximately 30–50% of cells were HCV-core-positive, while only 10–30% HCV-core-positive cells were found in FBS. In line with this, initial viral titres in CBS were also higher than in FBS. Higher infection rates might be facilitated through higher levels of HCV entry receptor SR-b1, as we had observed. Alternatively the infection cycle of HCV might be shorter in CBS-cultured cells, for example as a result of a more efficient secretion machinery, as was also

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Fig. 2. VLDL and albumin secretion. VLDL and albumin secretion was assessed in FBS-, HS- and CBS-cultured cells. Cells were washed extensively to remove all the albumin and VLDL that was present in the cell culture media, and the last wash was included in the measurements to determine background levels of either albumin or VLDL. We used an ELISA assay to measure albumin levels, and an FPLC-based method to determine lipoprotein profiles. (a) Neither FBS-cultured cells nor CBS-cultured cells secreted VLDL, in contrast to cells cultured in (adult) HS. (b) CBS-cultured cells secreted eight times more albumin than FBS cells, and the level of secretion was approximately the same as in fully differentiated, HS-cultured cells. Albumin secretion in cells cultured in adult bovine serum (ABS) was not increased. n≥3, expressed as mean with standard deviation for albumin measurements. **** indicates significant changes, P<0.0001. For VLDL secretion a representative profile is depicted.
observed for albumin. Core staining gradually increased to 70–80% in CBS media, compared with 40–50% in FBS at day 7 post-infection. More than 90% cells were positive both in CBS and FBS by day 10 post-infection (Fig. 3a). Thereafter, FBS cells underwent massive cell death [often referred to as the cytolytic event (Sainz & Chisari, 2006)], while CBS cells remained viable and maintained high percentages of infection (data not shown). Additionally, we measured levels of secreted HCV, by quantitating HCV RNA by quantitative PCR (Steenbergen et al., 2013). Our results showed that HCV RNA could be detected in the media of infected cells as early as 1 day post-infection, and the HCV (RNA) titres increased until day 5 in both FBS and CBS (Fig. 3b). However, beyond day 8–10 post-infection, HCV RNA levels decreased sharply in FBS, as a result of cell death. The typical drop in viral titres that was seen in FBS medium was not observed in CBS medium, and the viral titres remained stable for at least 60 days.

Summarizing, although Huh7.5 cells cultured in media containing CBS or FBS grew at similar rates, and morphologically were also comparable, culturing in CBS had a remarkable effect on albumin secretion, indicating better hepatocyte functionality, and was beneficial for the long-term production of HCV in cell culture as cells did not undergo cell lysis as a result of HCV infection.

Historically, foetal lysates or sera (e.g. chicken embryo lysates, foetal sera, cord blood sera) were added to tissue cultures to promote cell growth, because foetal materials are rich in growth-stimulating factors. CBS can be seen as the human equivalent of FBS because both of them are derived from blood taken at the highly proliferative phase of early development. Like FBS, CBS contains high levels of growth factors and low levels of antibodies, unlike their adult counterparts (Ang et al., 2011). Several growth factors and cytokines are increased in CBS compared with HS, including EGF (epidermal growth factor), members of the FGF family (fibroblast growth factor), HGF (hepatocyte growth factor), IGF-1 (insulin-like growth factor 1) and IGFBP1 (IGF-binding protein 1). All these growth factors are essential in the proliferative stage of primary hepatocytes after partial hepatectomy, and their defective regulation is involved in cancer progression (reviewed by Böhm et al., 2010; Michalopoulos, 2007; Taub, 2004). HGF is an initiator of hepatocyte proliferation, both in vivo and in cell culture, and also plays a role in the dissociation of tight junctions in that process, together with members of the FGF family (Nakamura et al., 2011; Ornitz & Itoh, 2001; Steiling et al., 2003). Both CBS and HS also contain higher amounts of certain growth factors compared with FBS, particularly IGF-1 (~40–80-fold increase) and IGFBP-1 (~15-fold increase). IGFBP-1 modulates the activity of IGF-1 and can prolong its action, and IGF-1 improves the differentiation of hepatocytic stem cells, as demonstrated by increased levels of albumin secretion (A yatolahi et al., 2011). We also observed higher levels of albumin secretion in our study, both in CBS and in HS, while they remained low in FBS and ABS, and the increased amounts of IGF-1 in both human sera may contribute to this increase.

HCV production in CBS-cultured cells is high (albeit still ~5–10-fold lower than in HS), and cells do not undergo the typical cytolytic event that is observed in FBS-cultured cells, which is associated with rapidly decreasing viral titres. This was indeed observed in our FBS cells when they were infected with a tissue-culture-adapted variant of JFH-1. The absence of this cytolytic event was also observed in Huh-7 and Huh7.5 cells that were growth arrested and (partially) differentiated with DMSO-containing media (Sainz & Chisari, 2006) and in cells that were cultured in HS (Steenbergen et al., 2013). Our current results show that the lack of the cytolytic event is not linked to growth arrest, as we initially assumed. The mechanism underlying the higher
viral production and prevention of cell lysis remains unclear, but (partial) differentiation may play a role in it. However, it is apparent that the use of CBS is beneficial for long-term virus production, particularly when cell proliferation is desirable.

Based on this study, we conclude that CBS is a suitable replacement for FBS, and is even beneficial in certain aspects. However, we recognize that the limited availability of CBS, or high cost of commercially available CBS, are factors that limit the practicality and broad application of using CBS for cell cultures. CBS is available commercially from select suppliers, or could be obtained from clinics, as umbilical cords are typically discarded as medical waste. The number of umbilical cords per se did not limit the amount of serum we could collect in this study, but rather (and for good reasons) the low priority serum collection had in the delivery room.

The outcomes of this study, however, provide new avenues to investigate the relationship between HCV replication and differentiation, or to further investigate which factors in CBS are responsible for the effects observed. Potentially, these factors could be added to FBS to improve cell cultures in general, or to avoid the HCV-induced cell lysis. Although we have not tested this, spiking FBS with CBS might achieve the same beneficial effects, without having to rely solely on CBS.

Summarizing, cells cultured in CBS proliferate at approximately the same rate as those in FBS and have increased levels of albumin secretion. Beyond that, CBS-cultured cells resemble FBS-cultured cells, in morphology, in mRNA levels that we determined and in the lack of VLDL secretion. Overall, our results show that culturing Huh7.5 cells in their allogeneic serum, in this case CBS, can be beneficial outside the stem cell research field, where allogeneic sera are already used regularly: the use of CBS is also beneficial for the long-term culture of HCV in cell culture because cells are able to continuously maintain high levels of virus production, without cell death.

References


