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Research paper

Increased exosome production from tumour cell cultures using the Integra CELLine Culture System ☆

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ABSTRACT

Exosomes are nanometer-sized vesicles, secreted from most cell types, with documented immune-modulatory functions. Exosomes can be purified from cultured cells but to do so effectively, requires maintenance of cells at high density in order to obtain sufficient accumulation of exosomes in the culture medium, prior to purification. Whilst high density cultures can be achieved with cells in suspension, this remains difficult with adherent cells, resulting in low quantity of exosomes for subsequent study.

We have used the Integra CELLine culture system, originally designed for hybridoma cultures, to achieve a significant increase in obtainable exosomes from adherent and non-adherent tumour cells. Traditional cultures of mesothelioma cells (cultured in 75 cm^2 flasks) gave an average yield of 0.78 μg ± 0.14 μg exosome/ml of conditioned medium. The CELLine Adhere 1000 (CLAD1000) flask, housing the same cell line, increased exosome yield approximately 12 fold to 10.06 μg ± 0.97 μg/ml. The morphology, phenotype and immune function of these exosomes were compared, and found to be identical in all respects. Similarly an 8 fold increase in exosome production was obtained from NKL cells (a suspension cell line) using a CELLine 1000 (CL1000) flask. The CELLine system also incurred ~5.5 fold less cost and reduced labour for cell maintenance.

This simple culture system is a cost effective, useful method for significantly increasing the quantity of exosomes available from cultured cells, without detrimental effects. This tool should prove advantageous in future studies of exosome-immune modulation in cancer and other settings.

1. Introduction

Exosomes are nanometer sized vesicles, which are actively secreted by most if not all nucleated cells. Their physiological functions are diverse, generally related to the functions of the parent cell (Johnstone, 2005), but most published reports relate to their immune-modulatory roles (Thery et al., 2002; Keller et al., 2006). Several studies have documented the immune-activating capacity of exosomes purified from antigen presenting cells (Raposo et al., 1996; Zitvogel et al., 1998), or mast cells (Skokos et al., 2001). The effect of exosomes from other cellular sources on immune responses is less well understood. This is particularly true for exosomes produced by neoplastic cells, where the literature represent apparent conflicting reports of immunological activation (Wolfers et al., 2001; Andre et al., 2002; Gastpar et al., 2005) or immune-suppression (Abusamra et al., 2005; Taylor and Gerçel-Taylor, 2005; Clayton et al., 2007) mediated by tumour exosomes.

Exosomes are produced by tumours in vivo, and are present in abundance in malignant effusions of advancing disease; such as ovarian carcinoma (Andre et al., 2002; Navabi et al., 2005) or pleural malignant mesothelioma (Bard et al., 2004). Although it has been possible to perform some investigations utilising such ascites-derived exosomes (Andre et al., 2002); doing so reliably and consistently

Abbreviations: CLAD, CELLine Adhere; CL, CELLine Classic; D_2O, Deuterium oxide.

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remains a challenge as it is difficult to obtain highly pure exosomes from this variable source material, which also contains non-exosomal serum-like protein and lipids (Bard et al., 2004), which may interfere with subsequent immunological assays. In contrast, obtaining highly pure exosomes from cell cultures should be more achievable, as cultures of homogeneous cell populations can be controlled to minimise potential contaminants in the purified exosome preparations. Unfortunately, however, the quantity of exosomes obtained from various culture systems to date is either very low, or requires scaling up of cell culture systems (e.g. roller bottle cultures) requiring the need to handle high volumes of culture medium. This represents a significant practical issue hampering progress in this research area.

Although there are several techniques available for exosome purification extensively detailed in (Théry et al., 2006), the most reliable method for obtaining high quantity and quality exosomes involves differential centrifugation, together with ultracentrifugation on sucrose gradients, as described (Raposo et al., 1996). Yet for purification of large quantities of exosomes, these methods are limited by the maximum capacity (volume of cell culture medium) of the ultracentrifuge being used. One approach to overcome this, may be to initially concentrate the culture medium, prior to centrifugation steps (Lamparski et al., 2002; Navabi et al., 2005). Our experience of such approaches (cross-flow ultrafiltration — (Navabi et al., 2005)), however, demonstrate that they are very time consuming and cumbersome to perform, and involve prohibitive costs for daily research use.

We have therefore adopted the integra CELLine culture system (originally designed for hybridoma culture) to achieve long-term high density cell growth (Trebak et al., 1999; Bruce et al., 2002; Favre et al., 2003). The CELLine system (Integra Biosciences AG, CH) is a two-compartment culture flask with a semi-permeable membrane around a concentrated cell-containing compartment allowing a constant source of nutrients from a larger outer medium compartment. Here we demonstrate that the cell culture medium in the cell compartment is approximately 12 fold enriched for mesothelioma-derived exosomes and 8 fold enriched for NKL-derived exosomes when compared to standard culture systems. Our method is useful for adherent and non-adherent cells, producing exosomes of comparable phenotype, morphology and immunological function to those obtained by standard cell culture systems. Further advantages are gained in terms of cost and investigator time in culture maintenance, and sample preparation.

We therefore advocate the use of the Integra CELLine system for exosome research, as an effective and simple means of significantly increasing the yield of exosomes from cultured cells.

2. Material and methods

2.1. Culture conditions

Cells were cultured in RPMI-1640 supplemented with L-glutamine (2 mM), Penicillin (100 U/ml) and Streptomycin (100 μg/ml), (from Cambrex) and 10% Foetal bovine serum (FBS), (from Gibco/Lifetechnologies Ltd). Bovine exosomes were removed from the FBS by ultracentrifugation at 100,000 ×*g* for 18 h, followed by serial filtration (0.22 μm followed by 0.1 μm) using vacufilter units (Millipore). In addition NKL cells were supplemented with 500 U/ml IL-2 (Proleukine) for optimal proliferation (Robertson et al., 1996).

Mesothelioma cells, obtained from pleural fluid of a patient with pleural malignant mesothelioma (referred to as mesothelioma throughout the manuscript), were seeded at 10^6 cells per flask (75 cm^2 vented culture flasks; Greiner) in a volume of 20 ml culture medium. Cells were grown in an incubator at 37 °C in 5% CO_2; after 1 week, the cells had reached 80–100% confluency. The cell-conditioned medium was harvested, and kept for exosome purification. Cells were split 1/2 into fresh flasks once a week, following treatment with 0.05% trypsin-EDTA (Gibco). Cells were routinely maintained at >90% viability (90.2%±2.42 viable (n=3)). In parallel, mesothelioma cells were seeded at an initial density of 25×10^6 cells in a 15 ml volume, into a CELLine AD (adhere) 1000 flask (CLAD1000) (Integra Biosciences AG). The outer "nutrient" chamber was filled with 500 ml culture medium. Cell-conditioned media were collected from the cell compartment each week. The cell compartment was subject to 3-washes in RPMI-1640, to remove any non-adherent or dead cells/debris, before addition of fresh FBS supplemented medium to both compartments. At three independent time points the adherent cells were harvested (following 0.05% trypsin-EDTA treatment) and subjected to trypan blue staining to assess cell death. Cells were routinely maintained at >90% viable (92.6%±0.97 viable (n=3)) and therefore, not significantly different to cells grown in the traditional cell culture system.

NKL cells are an NK (natural killer) cell clone established from the peripheral blood of a patient with large granular lymphocyte (LGL) leukaemia (Robertson et al., 1996). This cell line was obtained from the MRC cooperative (Cardiff University). NKL cells were seeded at 10^7 cells per flask (75 cm^2 vented culture flasks; Greiner) in a volume of 20 ml culture medium. Cells were grown in an incubator at 37 °C in 5% CO_2; after 4 days, had reached 80–100% confluency. The cell-conditioned medium was harvested, and kept for exosome purification. Cells were routinely maintained at >80% viability, verified by trypan blue staining (88.1%±2.1 viable (n=10)). In parallel, NKL cells were seeded at an initial density of 30×10^6 cells in a volume of 15 ml culture medium, into a CELLine 1000 flask (CL1000) (Integra Biosciences AG). As with the adherent system (CLAD1000) the outer "nutrient" chamber was filled with 500 ml culture medium. Cell-conditioned media were collected from the cell compartment every 4 days. The cell compartment was subject to 3-washes in RPMI-1640, to remove dead cells/debris before re-seeding the NKL cells at 40×10^6 cells in 15 ml medium. Cells were maintained at >80% viability (84.13%±2.01 viable (n=10)), again showing no significant difference to cells grown in the traditional culture system. Cell-conditioned media from each culture system was subsequently used for exosome purification, in the same way. Cell cultures were confirmed negative for mycoplasma contamination by monthly screening using the Venor ® GeM mycoplasma detection kit (Minerva Biolabs GmbH, Germany).

2.2. Exosome purification and quantification

Exosome purification was performed as previously described by a method based on the flotation characteristics

of exosomes on sucrose gradients, during ultracentrifugation. The method is effective at removing non-exosomal material which has a different density from exosomes (Thery et al., 2001; Lamparski et al., 2002; Clayton et al., 2007). Briefly, cell-conditioned medium was cleared of cells and large debris by serial centrifugation (400 ×*g* for 10 min then 2000 ×*g* for 15 min). Cell-conditioned medium was under-layered with a 30% Sucrose/D_2O cushion before ultracentrifugation at 100,000 ×*g* for 2 h (with a SW32 rotor, and an Optima LE80K Ultracentrifuge, Beckman Coulter). The collected sucrose cushion, was diluted in excess PBS and exosomes pelleted by ultracentrifugation at 100,000 ×*g* for 2 h (with a fixed angle 70Ti rotor, Beckman Coulter). Exosome pellets were typically resuspended in 100 μl PBS and stored at −80 °C. Exosome quantity was determined by the BCA protein assay (Pierce).

2.3. Electron microscopy

A method similar to that previously reported (Raposo et al., 1996) was employed to visualise exosome preparations by electron microscopy. Briefly, frozen exosomes were thawed on ice and resuspended in 1% glutaraldehyde in PBS (pH 7.4). A 5 μl drop of the suspension was transferred onto a pioloform-coated copper grid and allowed to stand for 5 min at room temperature. The grid was transferred to a 50 μl drop of double distilled water for 2 min and this process was repeated seven times for a total of eight water washes. The sample was stained on ice for 10 min with a 5 μl drop of 2% methyl cellulose containing 2% uranyl acetate. Excess fluid was removed with Whatman filter paper and allowed to air dry for 10 min before viewing by transmission electron microscopy (Philips EM 208, FEI).

2.4. Western blot analysis

Cell lysates were prepared from NKL and mesothelioma cells as positive controls for gp96 and calnexin analysis. Cells ($2–5\times10^6$) were lysed at 4 °C for 30 min in 500 μl of lysis buffer (containing 2% NP40 and 1× Complete protease inhibitor cocktail (Roche, GmbH)). Equal volumes of SDS-sample buffer (6 M Urea, 2% SDS, 30% glycerol, 50 mM Tris–HCL and freshly added 5% v/v β2 Mercaptoethanol) were added to purified exosomes/cell lysates (5 μg protein/well). Samples were subject to electrophoresis through 10% bisacrylamide gels, and transferred to PVDF membranes. Following overnight blocking in 3% non-fat milk in 0.5% Tween-20 in PBS (PBS-T), primary antibody (0.2–2 μg/ml) was added for 1 h, followed by three washes (PBS-T). Molecular weight markers (Cruz Marker™ Santa Cruz Biotechnology Inc.) and primary antibodies were detected using Cruz Marker™ compatible goat anti-mouse/goat anti-rat (where applicable) IgG-HRP conjugated at 1/12,000 dilution in PBS-T (Santa Cruz). Following 30 min incubation PVDF membranes were washed 5× in PBS-T. Bands were visualised using the ECL^+ system (GE Healthcare). The antibodies used (mouse anti-human monoclonal antibodies) were anti-TSG101, calnexin, hsp70, hsp90, hsc70, ICAM-1 (purchased from Santa Cruz). Anti MHC Class-I antibody (clone HC10) was obtained from the MRC-cooperative, School of Medicine, Cardiff University. Anti-CD81 antibody (also purchased from Santa Cruz) was used on samples prepared with non-reducing conditions. Anti-grp94 (gp96) antibody (rat anti-human monoclonal antibody) was purchased from Stressgen Biotechnologies.

2.5. Proliferation assay

Mesothelioma-derived exosomes have an anti-proliferative effect on T cells as described by Clayton et al. (2007). The T cell lymphoma cell line, Jurkat, was used to test the anti-proliferative function of the mesothelioma-derived exosomes (Taylor et al., 2006; Clayton et al., 2007). Jurkat cells were seeded at 10^5 cells per well in 200 μl complete RPMI-1640 supplemented with 5% FBS, in the presence of 250 U/ml Interleukin-2 (IL-2) with or without 1, 5 or 10 μg exosomes obtained either from traditional culture or from the CLAD1000 flask. Jurkat cells in 5% FBS only in the absence of IL-2 were used as an unstimulated control. The cells were incubated at 37 °C and 5% CO_2 for 3 days and pulsed for the last 18 h of culture with 1 μCi/well of ^{3}H-thymidine. Cells were harvested (Harvester 96, Tomtec) and the ^{3}H-Thymidine incorporation was measured (Microbeta-3, Perkin Elmer).

2.6. Statistical analysis

All data are represented as Mean±SE (Standard error). Comparisons between exosome treated and non-treated groups were performed using paired *t*-tests, calculated using Prism-4 (version 4.03), graphing and statistical software from Graph Pad, San Diego, CA. (Asterisks in figures represent;*$P<0.05$, **$P<0.01$, ***$P<0.001$).

3. Results

3.1. Exosome quantity is significantly elevated when using the integra CELLine culture system

Traditional 'flask' cell culture and the CLAD1000 flask were used for long-term culture of mesothelioma and other cells. Cell-conditioned medium was collected and processed for exosome purification each week over a period of approximately 300 days. The quantity of exosomes obtained was measured by the BCA protein assay and expressed as μg/ml of cell-conditioned medium. By evaluating the quantity of exosomes obtained from 20 preparations we observed a consistently higher exosome yield using the CLAD1000 flask compared to traditional culture systems. On average, there was a 12 fold increase in exosomes per ml of medium processed from 0.78 μg±0.14 μg/ml to 10.06 μg±0.97 μg/ml (Fig. 1A). When processing 180 ml of cell-conditioned medium, which is the maximum volume-capacity of the floor-standing Optima LE80K ultracentrifuge with a SW32 swing-out rotor, it was, on average ($n=13$), possible to purify 1.5 mg±0.05 mg of exosomes with the CLAD1000 flask compared with 0.15 mg±0.03 mg of exosomes when using traditional cell culture. Maintaining cells for 6, 7 or 8 days prior to harvesting conditioned medium from the CLAD1000 flask, revealed an accumulation of exosomes over time (Fig. 1B), demonstrating the advantage of feeding the flask infrequently to allow exosomes to effectively accumulate in the cell-containing compartment. It also confirms that exosomes are well trapped within this compartment (i.e. do

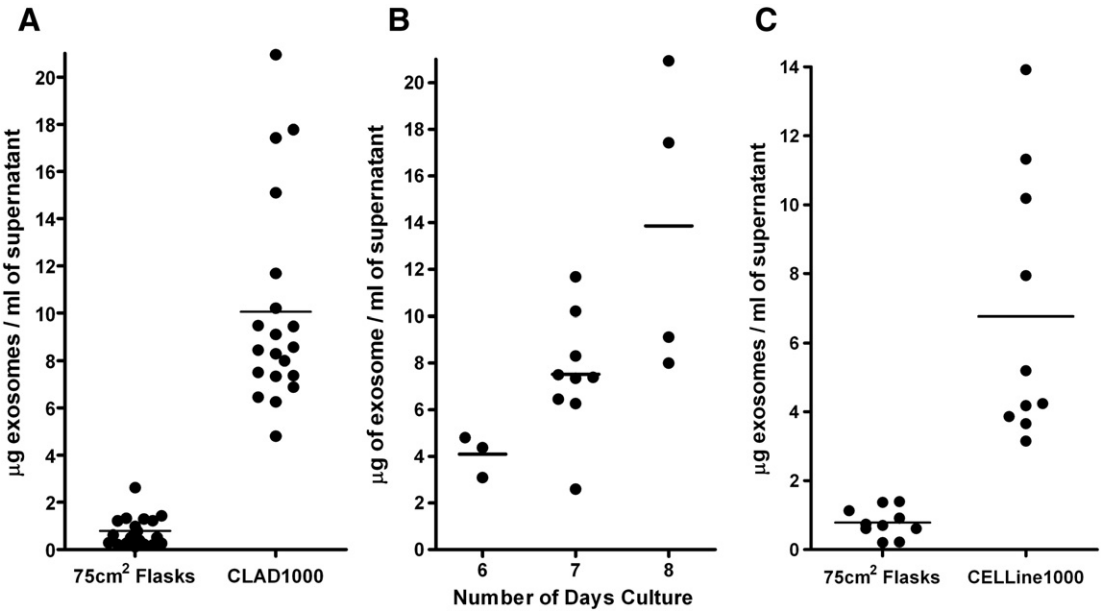

Fig. 1. Significant increase in exosome quantity using the CLAD1000 and CL1000 flasks. Mesothelioma cells were cultured in either traditional culture flasks (75 cm^2 flasks) or the CLAD1000 flask as described in Materials and methods. Cell-conditioned medium was removed weekly and processed for exosome purification. The yield of exosomes is expressed as μg/ml of culture medium. Here, 20 samples are shown for each culture system with an average 12 fold difference in yield (A). The amount of exosomes increased with the length of cell culture for the CLAD1000 flask (B). NKL-derived exosomes were enriched, by using the CL1000 flask, by an average of 8 fold across 10 preparations (C).

not traverse the semi-permeable membrane), and do not become degraded in situ during this time frame. Culture of the non-adherent NKL cell line using the CL1000 flask also produced significantly enriched exosomes compared with the traditional 75 cm^2 flasks. NKL-derived exosomes from 10 preparations were on average enriched 8 fold in the CL1000 flask from 0.79 μg±0.13 μg exosome/ml cell-conditioned medium to 6.76 μg±1.21 μg/ml (Fig. 1C).

3.2. Electron microscope images of exosomes

Exosomes purified from cell-conditioned medium of traditional cell culture or the CLAD1000 flask were visualised using transmission electron microscopy. Fig. 2 shows a representative field of both the traditional cell culture derived exosomes (A) and the CLAD1000 flask derived exosomes (B). The preparations were morphologically comparable, representing vesicular structures, somewhat heterogeneous in nature but with a diameter of less than 100 nm. Preparations were devoid of apoptotic bodies as described by Thery et al. (2001).

3.3. Phenotypic analysis of exosomes

Exosomes purified from either traditional cell culture or the Integra CELLine system were analysed by western blotting. Equal quantities of exosomes were added to each lane, and blots were stained for a variety of exosomal and non-exosomally associated proteins. The exosomes had comparable levels of each exosome associated protein examined regardless of the culture system employed

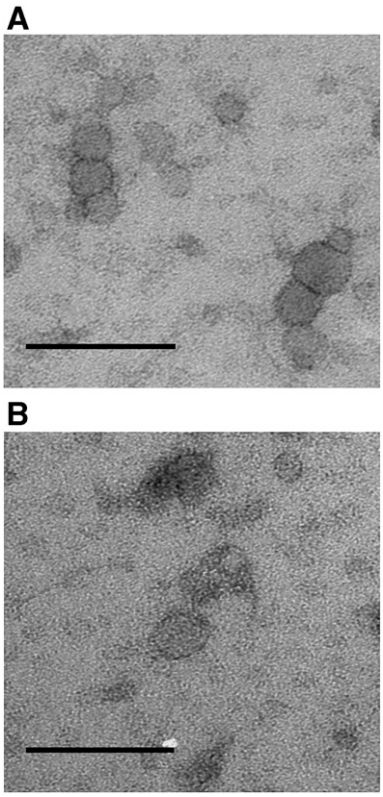

Fig. 2. CLAD1000 flask generates exosomes of comparable morphology. Mesothelioma-derived exosomes were visualised by transmission electron microscopy. The exosomes were obtained from either multiple traditional (75 cm^2) culture flasks (A) or from a CLAD1000 flask (B). The preparations exhibited comparable morphology, demonstrating a heterogeneous vesicular population of less than 100 nm in diameter (bar indicates 100 nm).

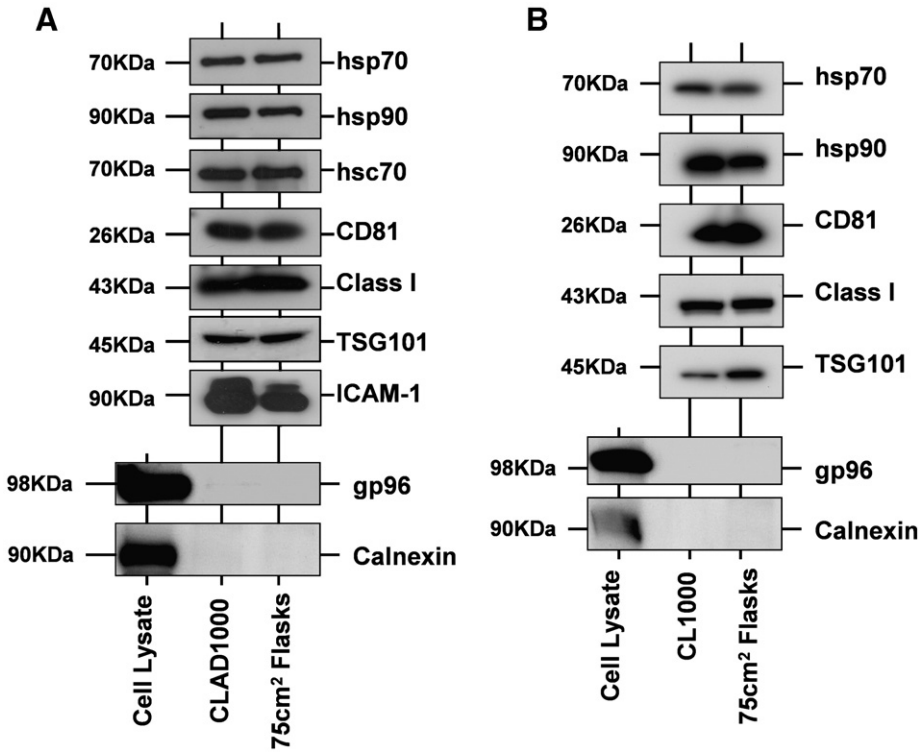

Fig. 3. CLAD1000 and CL1000 flasks generate exosomes of comparable molecular phenotype. Mesothelioma-derived and NKL-derived exosomes were purified from either multiple traditional cell culture flasks (75 cm^2 flasks) or from a CLAD1000/CL1000 flask respectively. Exosomes were quantified by protein assay and 5 μg exosomes, were subjected to SDS-PAGE and western blotting with antibodies as indicated above. Matched cell lysates were used as positive controls for antibodies to gp96 and calnexin. These western blots demonstrate that exosomes from the different culture methods express comparable amounts of the exosomal markers tested.

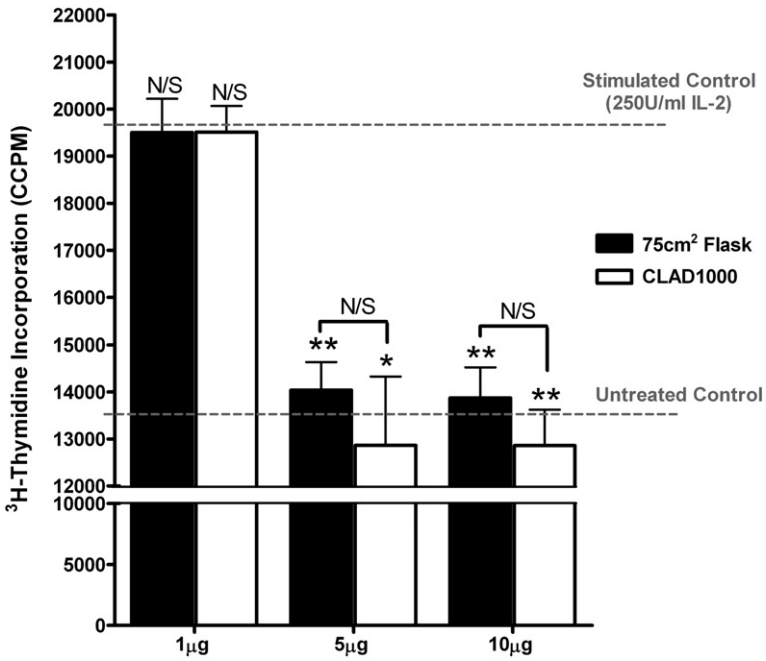

Fig. 4. CLAD1000 flask generates exosomes with comparable anti-proliferative capacity. Jurkat cells (10^5) were incubated for 3 days with 250 U/ml of IL-2 in the presence or absence of 1 μg, 5 μg or 10 μg of mesothelioma-derived exosomes; purified from either traditional cell culture (75 cm^2 flasks) or the CLAD1000 flask. Both exosome preparations significantly inhibited Jurkat proliferation at 5 μg and 10 μg but were not significantly different from each other (paired *t*-test).

(Fig. 3). This included similar levels of the multivesicular body-marker, TSG101; confirming there was no difference in the relative abundance of secreted-material originating from multivesicular bodies in these culture systems. Similar levels of other typically used markers (MHC Class-I, ICAM-1 and the tetraspanin CD81) were also evident, although there may be a small decrease in ICAM-1 within mesothelioma-derived exosomes from the CLAD1000 flask. Because heat shock proteins are dynamic, in terms of their cellular and exosomal expression levels, we also stained the blots with hsp-specific antibodies. The blots showed comparable levels of hsp70, hsp90 and Hsc70. This was important, as it indicates that cells cultured in the CLAD1000 and CL1000 flasks, were not experiencing undue cellular stress such as hypoxia, or starvation, and that the exosomes obtained from this system were phenotypically identical to those of traditional flasks. Exosome purity was further confirmed by the absence of the endoplasmic reticulum markers gp96 and calnexin in all exosome preparations, where as these markers were abundant in cell lysates from the same cells (Fig. 3). This again demonstrated the CLAD1000 and CL1000 flasks to be comparable to traditional cell cultures in terms of the level of non-exosomal contaminants in the exosome preparations.

3.4. Comparing exosome immune-modulatory function

Tumour exosomes, of various types, can inhibit the proliferative response of lymphocytes to various mitogens (Dukers et al., 2000; Taylor and Gerçel-Taylor, 2005; Clayton et al., 2007). To compare the functional efficacy of mesothelioma-derived exosomes obtained from each culture system, Jurkat cells were treated with IL-2 in the presence and absence of these tumour exosomes, and their proliferative response was assessed after 3 days. In the absence of mesothelioma-derived exosomes, Jurkat cells responded strongly to IL-2. Adding 5–10 μg exosomes (per 10^5 Jurkat cells) at the same time as IL-2 resulted in a significantly impaired proliferative response (Fig. 4). Importantly, however, this inhibition was achieved equally well with exosomes obtained from a CLAD1000 flask compared with traditional culture. The data show tumour exosomes, purified from cells cultured in the CLAD1000 flask, fully retain their anti-proliferative capacity.

4. Discussion

There has been a notable increase in recent years in research articles detailing the composition and biological functions of exosomes. Many reports describe the capacity of exosomes to modulate immune or inflammatory responses in the context of health, such as pregnancy (Taylor et al., 2006), or disease, such as arthritis (Zhang et al., 2006) and cancer (Taylor and Gerçel-Taylor, 2005; Liu et al., 2006; Clayton et al., 2007). Some manuscripts describe exosomal immune-functions even in immune privileged sites such as the central nervous system (Potolicchio et al., 2005) or the eye (McKechnie et al., 2003). Yet despite these advances, there remain many unanswered questions regarding the functions, and underlying mechanisms, by which exosomes mediate their complex biological effects. Key to the continued progress of this important research area is the ability to obtain sufficient exosomes for study, whatever the cell type of interest, a simple issue which remains a significant obstruction to progress in the field.

The Integra CELLine culture system was designed to assist monoclonal antibody production, by allowing high density, high viability culture of antibody-secreting hybridomas (Trebak et al., 1999; Bruce et al., 2002; Favre et al., 2003). We have adopted this approach to obtain high quantities of exosomes using the CLAD1000 flask for the Mesothelioma cell line and the CL1000 flask for the NKL cell line. The advantage is that cells are maintained at good viability (comparable to that of traditional flask culture) in a low volume of culture medium. The cells are trapped in a semi-permeable membrane, with a 10 KDa molecular weight cut off. This membrane allows passage of nutrients and metabolic waste products across the membrane, whilst trapping cell-derived secretory products, including exosomes, in the 15 ml cell compartment. This 15 ml of culture medium contains a comparable quantity of exosomes to that present in ~180 ml of traditional flask-culture medium (the maximum capacity of a typical floor-standing ultracentrifuge like the Optima LE80K, Beckman Coulter); therefore the exosome purification process is easier and quicker to perform, and also allows for better removal of non-exosomal protein. In addition, the CLAD1000 flask proved significantly more cost effective than traditional cell culture (Table 1) making the overall production of exosomes 5.5 fold cheaper. The data presented here stem from a CLAD1000 flask, which was seeded with 25×10^6 cells over 12 months ago. This flask is still producing high quality, high yield exosomes, and has provided over the study period a total of 8.575 mg of exosomes. Trying to achieve this quantity by traditional culture would involve ultracentrifugation of approximately 11,000 ml, and this is practically unviable.

Whilst the approach described here may not be suitable for all cell types, we have found the Integra culture system versatile, supporting long-term growth of diverse tumour cell types, from adherent mesothelioma or prostate cancer cells

Table 1
Costing of 75 cm^2 flask culture vs the CLAD1000 flask culture

	Investigator time/week	Flask cost/year	Reagent cost/year	D_2O cushion	Centrifuge tubes	Total cost	Exosome yield	Exosome cost
75 cm^2 flasks	1.5 h	€ 287	€ 141	€ 2325	€ 1794	€ 4547	0.78 μg/ml	€ 0.623/μg
CLAD1000 flask	0.7 h	€ 129	€ 380	€ 194	€ 172	€ 875	10.06 μg/ml	€ 0.112/μg

This table shows the projected costs, for each culture system employed, over 1 year and relates the cost to exosome yield. One CLAD1000 flask was used for the year where ten 75 cm^2 flasks were used per week. Reagents include medium and supplements, such as FBS. The D_2O cushion and ultra-centrifuge tubes are listed separately to highlight the inherent cost of processing large volumes through the ultra-centrifuge. The cost/μg of exosomes are related to the total amount of cell-conditioned medium processed and average exosome yield. Exosomes purified from the CLAD1000 flask were approximately 5.5 fold cheaper to produce than exosomes from traditional cell culture.

(DU-145 and LN-CAP) to suspension cells including the myelogenous leukaemic line K562, EBV-transformed B cells and the natural killer cell line-NKL. All attempts to date have achieved significantly increased exosome yield when compared to traditional cultures in 75 cm^2 flasks or various culture-plates. We have outlined the general protocol for tumour exosome production using the Integra CELLine culture system in the form of a flow diagram (Fig. 5). We feel that this would be a useful starting point for any group wishing to produce tumour exosomes, using the technique described in this manuscript, from their tumour cell line of choice.

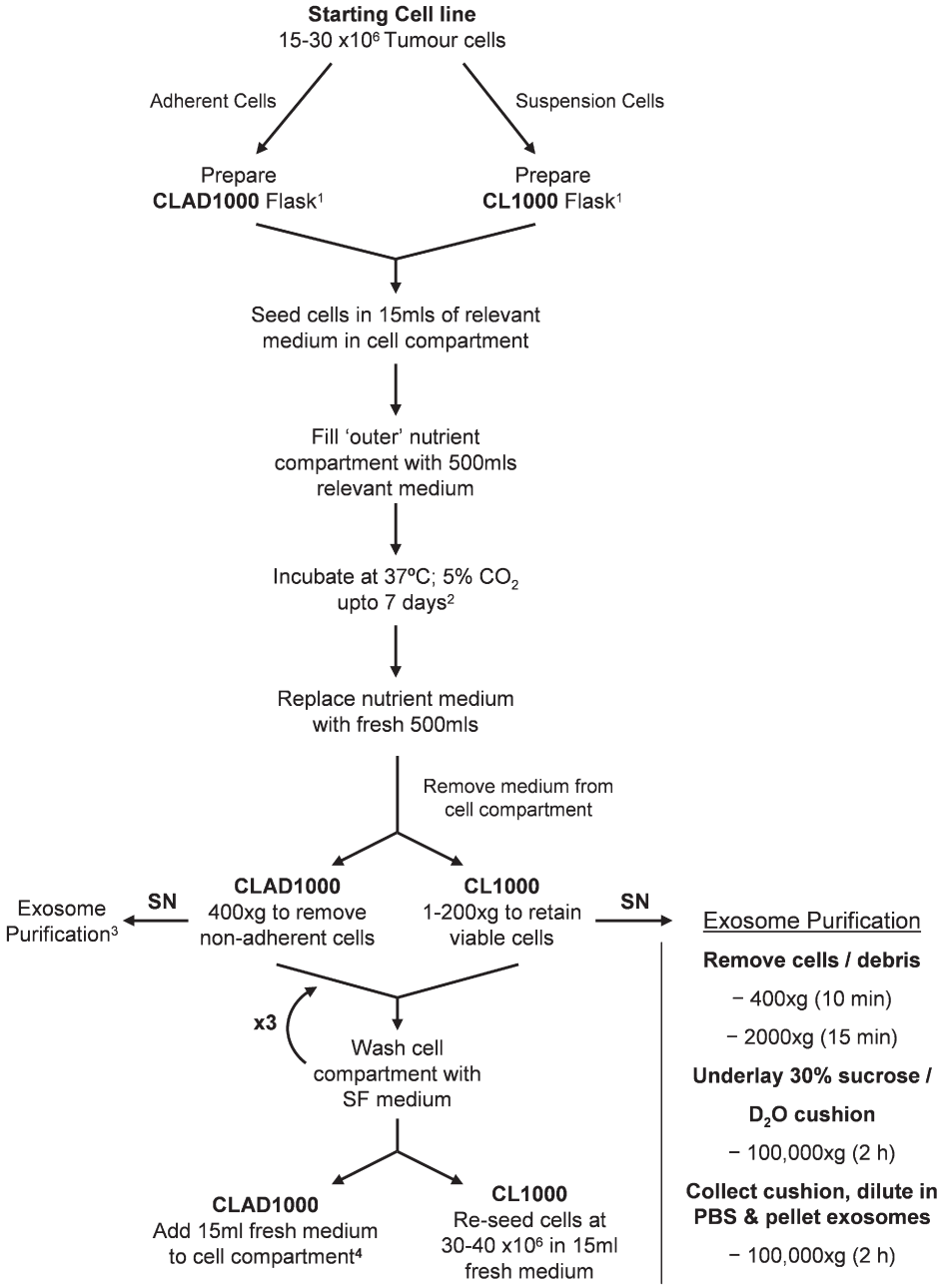

Fig. 5. CELLine Culture system protocol for tumour exosome production. This figure details the general protocol devised in this manuscript for tumour exosome production using the CELLine culture system. This protocol should be the starting point for researchers wishing to use the system for their tumour cell lines of interest and not as a definitive protocol for any cell line. The following abbreviations were used SN (supernatant), SF (serum free) and PBS (phosphate buffered saline). [1]The preparation of the flasks for cell culture should follow the manufacturer's instructions. [2]Flask incubation time is dependant on the cell type and growth kinetics and should be tailored for the cells used in each case independently. [3]Exosome purification was as described in the opposite arm. [4]In general it was found that trypsinisation was not necessary for the adherent cells used in this study. However, cell harvesting through trypsin treatment can be performed periodically, if required, and maybe a useful tool for checking the 'cell makeup' of a CLAD1000 flask.

Although we have largely followed manufacturer's guidelines when maintaining suspension cells in these flasks, we have modified the maintenance approach for adherent cells growing in CLAD1000 flasks. Specifically, the manufacturers recommend routine harvesting of the cells by trypsin-EDTA treatment, which allows the number of cells in the flask to be counted, and viability assessed, prior to re-seeding. In our experience, the trypsin-digest process was not essential. We expected that with time, protein or particles may build up and cause problems in nutrient/waste/gas transport across the membrane. However, by vigorous washing of the cell compartment, poorly adherent or non-viable cells were effectively removed each week, without having to trypsin-treat the flasks. This method also avoids the accumulative damage to cells (with associated phenotypic change) that often occurs with increasing passage number, due to repeated trypsin-based harvesting. This seemed sufficient to prevent build up of material on the cell-compartment membrane, as the flasks were successfully maintained for >6 months for each cell line we have used. At intermittent time points it maybe necessary to assess cell number and viability within the CLAD1000 flask, using trypsin-EDTA to harvest the adherent cells. This infrequent trypsin-based harvest maybe a compromise to repeated treatments, thus giving the researcher peace of mind and still reducing damage caused by repetitive trypsin treatment. However, following the mesothelioma-derived exosome yield over time from the CLAD1000 flask we observed little decrease with time, strongly suggesting that the flasks have reached a saturating cell dose, which is largely stable.

In conclusion we advocate the use of the 'Integra CELLine Culture System' for high yield/high grade exosome production at low cost and time to the user.

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