In vitro CMV-infection model in fresh and glycerolized skin graft

Sara Astegiano¹, Daniela Alotto², Carlotta Castagnoli¹, Franca Sinesi¹, Maurizio Stella², Massimilano Bergallo¹, Rossana Cavallo¹

¹Virology Unit, University Hospital San Giovanni Battista di Torino, Turin, Italy; ²Department of Reconstructive Plastic Surgery, Burns Centre and Skin Bank, Trauma Center, Turin, Italy

SUMMARY

Viral infections, especially cytomegalovirus (CMV), are a cause of death in burned patients. Aim of this study was to perform an in vitro CMV-infection model comparing fresh and glycerol-treated fibroblasts and keratinocytes. Cells were plated in plates for the two conditions. Each plate was set up with CMV dilutions. Immunofluorescence and real time PCR assays were performed. The assays were negative in both fresh and glycerolized keratinocytes. For fibroblasts, CMV-DNA was positive in both conditions and immunofluorescence test only in fresh cells. Glycerol at 85% confirms its strong virucidal effect as reported also for other viruses.

KEY WORDS: Human cytomegalovirus, Skin graft, Infection

Historically, bacterial pathogens have been the most common cause of infections in burns patients (Limaye et al., 2008). However, the widespread use of topical anti-microbials has resulted in the decline, even if not the elimination, of bacterial wound infections (Mayhall, 2003). In addition to infections caused by bacteria and fungi, there are data implicating viral infections as a cause of death among severely burned patients, especially herpesviruses such as herpes simplex virus (HSV) and cytomegalovirus (CMV) (D’Avignon et al., 2003). In particular, CMV has long been recognized as an important viral pathogen in immunocompromised hosts. In addition to direct effects due to viral replication and resulting tissue injury, a range of indirect effects have been attributed to CMV in immunocompromised patients (Pérez-Sola et al., 2008). Among them, burns patients are particularly susceptible to infections and sepsis due to the impairment in the functional capacity of T cells, as well as to the loss of the cutaneous barrier. Cadaveric skin is widely used to treat these patients (Rennekampff and Hamprecht, 2006). In fact, allografts offer many advantages in wound coverage, including that of being able to create a physiological barrier, which greatly decreases the loss of water, electrolytes, proteins and heat, through the wound (Obeng et al., 2001). They also create a mechanical barrier which protects the lesion from random environmental bacteria contamination (Alotto et al., 2002).

Long-term preservation of human skin can be obtained successfully by cryopreservation and glycerol preservation. Cryopreservation offers viable tissue after thawing, whilst glycerolized tissue is not viable but maintains its structural and mechanical properties (Castagnoli et al., 2003). In our previous study we evaluated the prevalence of CMV-DNA in donor skin biopsies comparing fresh and glycerolized samples (Astegiano et al., 2010). We noted that in vitro studies were necessary to determine the effect of glycerol on intracellular viral survival to consider the implications for the clinical use of fresh or glycerolized preserved skin in burns patients. For that reason, this study describes an in vitro CMV-infection
model in fresh cutaneous fibroblasts and keratinocytes, in comparison with the same cells treated with different concentration of glycerol, in order to mimic the conservation procedure for skin grafts used in skin banks for the treatment of burned patients. The different phases of the protocol were performed in two different facilities: a ‘sterile’ zone, in particular the skin bank laboratory of the CTO Hospital of Turin, for the cultivation of dermal fibroblasts and keratinocytes, and an ‘infective’ zone, the virology unit of the San Giovanni Battista University-Hospital of Turin, for CMV infection, the immunofluorescence assays and the molecular tests.

Human split thickness skin grafts (0.4 mm thick), derived from a subject with CMV-seronegative status, were incubated with 2 mg/ml dispase II (Roche Manheim, Germany) O/N at 4°C. After incubation, dermal and epidermal layers were mechanically separated. Epidermal layers were incubated with trypsin 0.05% (Invitrogen Life Technologies, USA) for 10 min at 37°C in 5% CO₂ atmosphere to obtain keratinocyte cell suspensions.

The cells, plated at 1.6x10⁴ cells/cm² density, were grown on a irradiated 3T3-J2 layer, as described by Rheinwald and Green (1975), in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, UK) and 10% fetal bovine serum (FBS, New Zealand origin) supplemented with 5 µg/ml insulin (Sigma-Aldrich, USA), 0.4 g/ml hydrocortisone, 10⁻¹⁰ M cholera toxin, 2x10⁻⁹ M tri-iodothyronin, 5 µg/ml apo-transferrin (Invitrogen Life Technologies, USA), 2 mM glutamine, and 50 U/ml penicillin-streptomycin antibiotic solution. The flasks were incubated at 37°C in 5% CO₂ atmosphere to obtain keratinocyte cell suspensions.

Primary cultures of human keratinocytes were grown to approximately 80% confluence after about fourteen days. The cells were enzymatically separated by digestion with 0.05% trypsin (Invitrogen Life Technologies, USA). Residual 3T3-J2 were removed before harvesting by washing with 0.05% trypsin. The recovered keratinocytes were re-washed, re-suspended in culture medium and plated out in the 6-well plates at a density of 5x10⁵ cells/well. Overall, 4 plates were set up, 2 for the fresh condition and 2 for the treatment with glycerol and used in parallel for CMV-infection cellular model and molecular test (CMV-DNA detection). Dermal layers (12 six-millimetre punch biopsies) were incubated in a 12-well plate with Dulbecco’s Modified Eagle Medium (DMEM, Gibco, UK) and 10% fetal bovine serum (FBS, New Zealand origin) for about seven days at 37°C in 5% CO₂. Every three days the medium was changed. After this period, the biopsies were removed. The wells at confluence, in which fibroblasts replicated, were incubated with 0.05% trypsin, were re-suspended in culture medium and plated out in 75 cm² tissue culture flasks (Sacco). Fibroblasts at third/fourth passage and at 80% of confluence were enzymatically separated by digestion with 0.05% trypsin, re-suspended and plated out in six-well plates at a density of 2.5x10⁵/well. Overall, 8 plates were prepared, 4 for the fresh condition and the others for treatment with glycerol and used as describe for the keratinocytes.

For the CMV-infection model, dilutions from the CMV AD169 strain were performed. Each plate set up was as follows: one well for the negative control (only culture medium) and the others for CMV dilutions 10⁻², 10⁻¹, 10⁻⁰, 10⁰, 10¹ TCID₅₀. When cytopathic effect (CPE) was evidenced about 10 days post-infection (p.i.) cells from each well were digested with trypsin, re-collected and centrifuged. In the case of the fresh condition, centrifuged cells were immediately divided into two aliquots, one half used for CMV-DNA detection by the real time PCR assay as previously described (Astegiano et al., 2010) and the other for shell vial infection. Briefly, shell vial assay was performed by inoculating the human embryonic lung fibroblasts (HEL-F) monolayer with 200 µl of supernatant obtained from in vitro culture of fibroblasts or keratinocytes infected with CMV. Shell vials were centrifuged at 1500 rpm for 45 min and incubated for 48 hours at 37°C in a 5% CO₂ atmosphere. Then shell vials were fixed with methanol-acetone and indirect immunofluorescence was performed, using commercial primary monoclonal antibody (clone E13, diluted 1:50 in PBS 1X, 1% albumin) and a goat antimouse IgG+IgM FITC conjugated (diluted 1:100 in PBS 1X, 1% Evans blue) as secondary antibody (Argene, Biosoft, France).

On the other hand, cells for glycerol treatment were centrifuged and incubated with different...
concentrations of glycerol, made up in phosphate-buffered saline (PBS): 50% of glycerol for 3 hours, 70% for 3 hours and preserved for almost two weeks at 85% of glycerol, always at 4°C, mimicking the procedure of the treatment of skin graft for transplantation used in the CTO skin bank.

After this treatment the experimental protocol was the same as that of fresh cells. Results are summarized in Table 1. Overall, for each condition immunofluorescence test and real time PCR assay were performed in duplicate. Considering the keratinocytes, at 10 days p.i all the wells infected in vitro with CMV dilutions were tested in real time PCR and all resulted negative in both fresh and glycerolized cells. In order to confirm these data the immunofluorescence test was performed and all were negative in the case of fresh condition but it was not possible to determine in the case of glycerol-treated cells because of a partial destruction of the monolayer of cells in the shell vial support.

As regards the dermal fibroblasts, the CPE effect was clearly evidenced only in 10^5 and 10^3 TCID_{50} wells after 10 days p.i. In the case of the other dilutions CPE effect was not noticed, probably because low dilutions needed more incubation time p.i., until 21 days.

This is why the immunofluorescence assay, in the case of fresh condition, was positive only for the high CMV dilutions. In glycerolized cells the immunofluorescence test was negative. Molecular test was positive to CMV-DNA only for 10^3 and 10^5 TCID_{50} in both fresh and glycerolized cells and negative for the other dilutions and the negative control.

Human skin allografts are important tools in the treatment of severe burns. It is commonly recognized that the skin grafts must be processed and stored to maintain their viability and the structural integrity (Bravo et al., 2000).

Cryopreservation and glycerol preservation are the most common methods used. Glycerolization is a method developed by the Euroskin Bank (Beverwijk, The Netherlands) and consists in drying the skin by removing water from cells using increasing concentrations of glycerol, and glycerolized allograft (GPA) is finally stored in 85% of glycerol solution. Cryopreservation consists in the using of a controlled freezing process with compounds such as dimethylsulfoxide Me(2)SO (DMSO) or glycerol as cryoprotectant and storage in liquid nitrogen.

Glycerolized treatment destroys the vital structures, so that the allograft is considered non-viable, but preserves the morphology of the tissues, whereas cryopreservation allows for a certain level of viability after the tissues are thawed (De Backere, 1994; Eberwein-Blome et al., 2002; Hermans, 2011; Saegeman et al., 2008).

Biological dressings have intrinsic antimicrobial properties in different levels and in vitro studies indicate that, among other factors, these antimicrobial effects depend on preservation methods of

### Table 1 - Results of immunofluorescence and molecular tests.

<table>
<thead>
<tr>
<th></th>
<th>Fresh Keratinocytes</th>
<th>Glycerolized Keratinocytes</th>
<th>Fresh Fibroblasts</th>
<th>Glycerolized Fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunofluorescence test</td>
<td>ctrl neg: N</td>
<td>ctrl neg: N.D.</td>
<td>ctrl neg: N</td>
<td>ctrl neg: N</td>
</tr>
<tr>
<td>10^2: N</td>
<td>10^2: N.D.</td>
<td>10^2: N</td>
<td>10^2: N</td>
<td>10^2: N</td>
</tr>
<tr>
<td>10^5: N</td>
<td>10^5: N.D.</td>
<td>10^5: N</td>
<td>10^5: P</td>
<td>10^5: N</td>
</tr>
<tr>
<td>Real-time PCR assay</td>
<td>ctrl neg: N</td>
<td>ctrl neg: N</td>
<td>ctrl neg: N</td>
<td>ctrl neg: N</td>
</tr>
<tr>
<td>10^2: N</td>
<td>10^2: N</td>
<td>10^2: N</td>
<td>10^2: N</td>
<td>10^2: N</td>
</tr>
<tr>
<td>10^4: N</td>
<td>10^4: N</td>
<td>10^4: N</td>
<td>10^4: N</td>
<td>10^4: N</td>
</tr>
<tr>
<td>10^5: N</td>
<td>10^5: N</td>
<td>10^5: N</td>
<td>10^5: P</td>
<td>10^5: P</td>
</tr>
</tbody>
</table>

N: negative; N.D.: not determined; P: positive.
the skin grafts (Hermans, 2011). Cryopreserved allografts may be a potential vector for bacteria or virus from donors to recipients, as may occur in other organ and tissues transplantation (Hermans, 2011), and suspected transmission of HIV (Clarke, 1987) and CMV-seroconversion (Kealey GP et al., 1996) have been noted. Different studies (Van Baare et al., 1998; Marshall et al., 1995) have reported a significant percentage of cryopreserved allografts discarded for positive cultures and/or serology, either from donors and the different preservation methods were found in bacteria, such as infection in dermal fibroblasts and epidermal keratinocytes are not permissive for CMV replication.

In this study we devised an in vitro model of CMV infection in dermal fibroblasts and epidermal keratinocytes, taking into account different conditions, fresh and glycerolized cells, mimicking the treatment of allograft skin biopsies used in skin banks. Our results on keratinocytes demonstrate that infection with CMV was absent after 10 days p.i. This result is probably due to the fact that keratinocytes are not permissive for CMV replication. On the other hand, glycerolized fibroblasts seem to confirm previous studies on glycolal antigenic activity: in fact, in fresh infected cells the immunofluorescence was positive at $10^3$ and $10^5$ TCID$_{50}$, while, in glycerolized infected cells, positivity was not found. Further studies will be necessary to confirm these data. Although no comparative randomized studies for the different preservation methods were found in literature, lower cost due to the minimal storage requirements (a household refrigerator) and superior intrinsic antimicrobial activity are the main features that seems to favour glycerol preservation over cryopreservation.

REFERENCES


immunocompetent patients. *JAMA.* **300**, 413-422.


