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Obtaining 3D and 3D revert cultures of BMG-1 cell line for the analysis of cytokine expression differences

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Methods to utilize cell lines as research material are evolving continuously as with the parallel advancement in instrumentation and analysis technologies. One such advancement in culture methodology of particular significance is the 3-dimensional (3D) way of culturing cells. It is now clear that 3D cultured cells behave differently from their monolayer (2D) counterparts and provide meaningful insights into complex cellular mechanisms that are rather difficult to study using 2D cultured cells. We take a step further and describe 3D-reverts, an extension of the '2D to 3D' culture methodology. We demonstrate that 2D, 3D and 3DRs express cytokines differently and also that such differences extend to the culture stages of 3D and 3DRs, in a time-dependent manner. This approach of analysing differences between 3D and 3DRs as a time-dependent or culture stage-dependent manner will surely enhance the utility of cells that will augment the 3D culture systems.

Keywords: Agarose hydrogels, BMG-1, 3D aggregates, 3D reverts, cytokines; differential expression.

CYTOKINES are mediators for several functions including those involved in complex immunological mechanisms as associated with several cell types. The functions of these mediators are even more significant in conditions such as cancers. Several networks of cytokines are known to be associated with specific cancer types, apart from a few individual ones that mediate specific functions in cancers[1,2].

The utility of cancer cell lines as material for cancer research has been greatly enhanced by culturing them as 3D aggregates/spheroids/tumeroids. This approach has resulted in obtaining meaningful results from the cultures and can be more relevant to a realistic *in vivo* condition[3,4]. We have taken a step further and looked into 3D reverts (3DRs) for their utility in cancer research. Such reverts can be obtained by reintroducing 3D cultures into culture units sans matrices or scaffolds. We feel that these reverts behave differently compared to their 2D and 3D counterparts whose analysis can provide insights into the complex mechanisms of cancer cell biology with a better resolution. In this study, we use BMG-1 (human malignant glioma) cell line to demonstrate that

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monolayers, 3D aggregates and 3DRs have different cytokine expression profiles. BMG-1 is a human brain malignant glioma cell line and has a doubling time of 24 h. Cytokines play important roles in the glioma growth and invasion. Some cytokines such as IL-6, IL-8, IL-1β, IL-10, IL-2, VEGEF, and TNF-α promote tumour progression, proliferation, and aggressive behaviour and are also involved in pathogenesis of the disease associated with pain[5,6]. We have recently reviewed the contributions of 3D cancer cell cultures for understanding cancer cytokines[7].

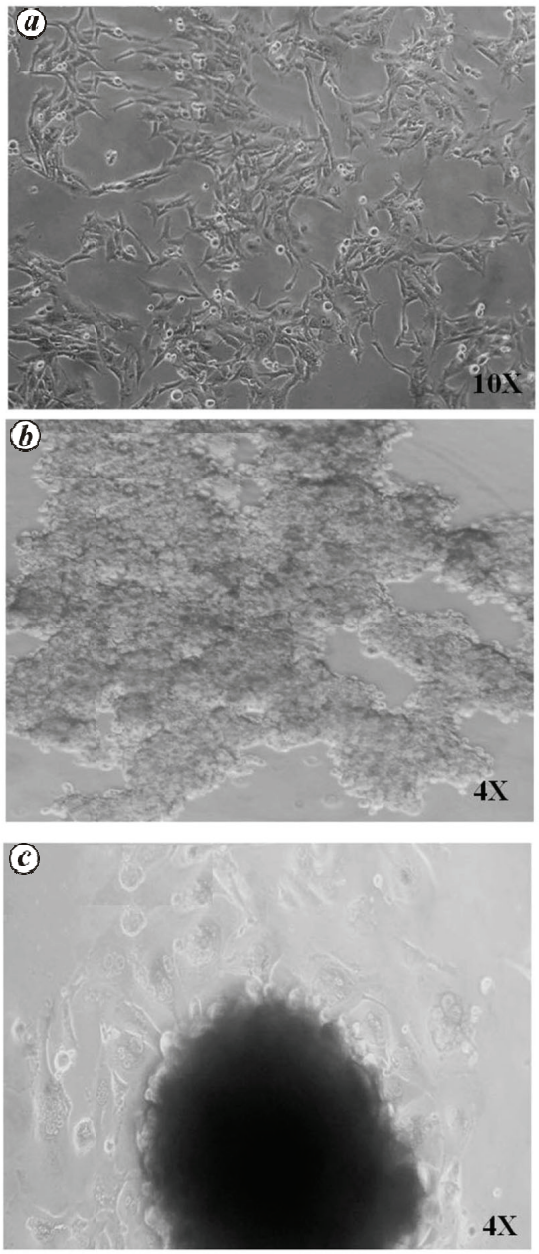

Figure 1. BMG-1 cell line monolayers (***a***) formed floating aggregates on 1% agarose hydrogels (***b***). They began to form 3DRs within a few hours of plating into wells devoid of the hydrogel (***c***).

We feel that the cell culture methods described, especially those involving the 3DRs, is a step further to currently utilized approaches in cell culture techniques. Several assays such as wound-healing and cell migration can be applied to the 3DRs as they can be relevant to cancer research.

The BMG-1 cell line was cultured as monolayers in T-25 flasks using DMEM supplemented 10% FBS (complete medium) (Figure 1 *a*). The cells were trypsinized at 95% confluency for obtaining 3D aggregates using 1% agarose hydrogel as the matrix with a seeding density of 5×10^4 cells/ml. The 1% agarose hydrogel prepared in DMEM without the serum was optimally heated to obtain a homogenous gel (complete melting of the agarose), and 1 ml of such molten gel was poured into each of the wells of the 12-well plates. The plates were then exposed to UV within a laminar flow chamber which served to polymerize and to sterilize the agarose hydrogels as required for obtaining the 3D cultures. One ml of the complete medium was added into each of these wells prior to seeding the cells.

Healthy floating aggregates at day-5 of culture were harvested along with the supernatants by gentle pipetting and transferred into 12-well culture plate wells in the absence of agarose hydrogels. This enabled reattachment of cells that comprised of the floating 3D aggregates to the culture plates, forming 3DRs.

Supernatants for cytokine profile analysis were harvested from 95% confluent healthy monolayers during days 1–10 of 3D aggregate and days 1–5 of 3DR cultures. These supernatants were analysed for a panel of cytokines using the ELISA kit supplied by R&D systems and the results were analysed using PRISM graphpad.

The cells formed healthy aggregates on day-5 of cultures, with the best culture morphology (Figure 1 *b*). The 3DRs started forming immediately after introducing them into wells sans the agarose hydrogels and the formation of explant-like monolayer spread continued till day-5 (Figure 1 *c*).

Of the cytokines profiled, a marked change in the levels of cytokines secreted into the culture supernatant was observed among the 2D and progressive 3D aggregates, except for the TGF-B. The peak levels of cytokine secretion were observed on day-2 of 3D aggregate culture for the cytokines TNF-α, IL-1β, IL-1ra, IL-10, IL-6 and IL-9. IL-27 and IL-17 were found to peak on day 3 of 3D aggregate formation and SDF-1 was found to peak on day-8. Among these, the expression of IL-1ra, IL-17, IL-9, IL-1β, TNF-α, IL-10, IL-6 and IL-27 was found to be significantly elevated in 3D cultures compared to their 2D counterparts.

The 2D and 3DRs showed similar profiles for cytokines IL-6, IL-1ra, IL-10 and TGF-β. Significant differences were seen for expression of cytokines SDF-1, IL-1β, TNF-α, IL-27, IL-17 and IL-9 among 2D and 3DRs. These similarities and differences peaked as the

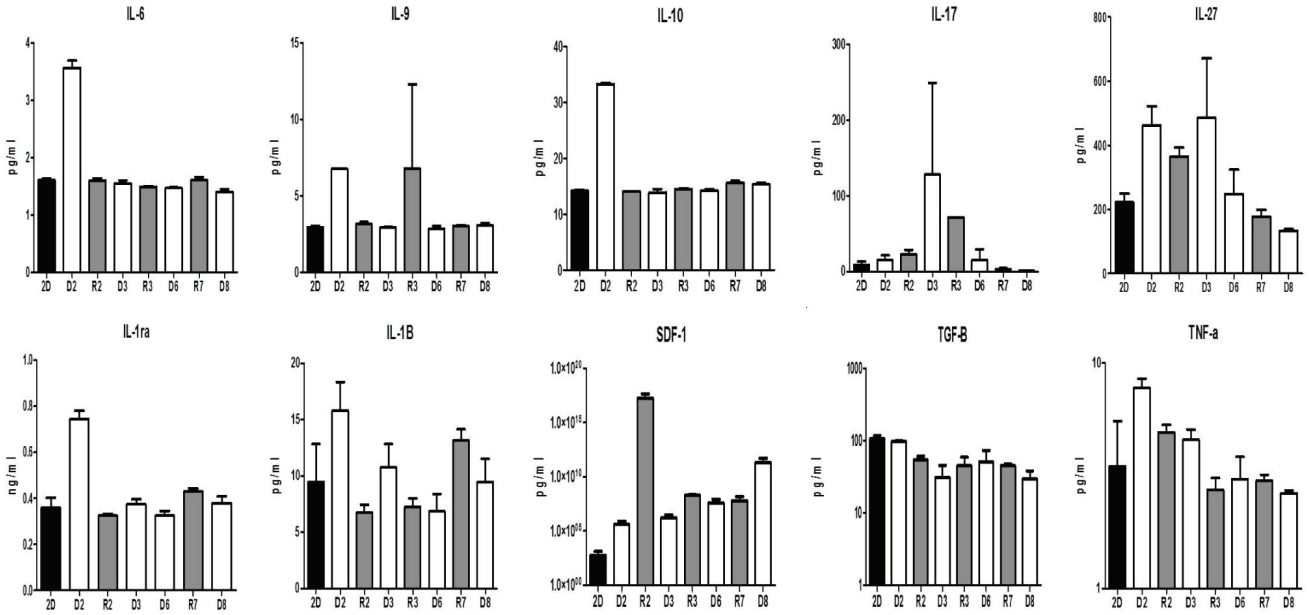

Figure 2. The 2D, 3D and 3DRs showed differences in the expression levels of 9 cytokines, the IL-6, IL-9, IL-10, IL-17, IL-27, IL-1ra, IL-1B, SDF-1 and TNF-α. Enhanced differences were observed on day-2 of 3D cultures when compared to the 2D monolayers indicating a fairly immediate response as induced by the 3D aggregate formation. Minimal differences in the expression levels of IL-6 and IL-10 were noticed as the 3D and 3DR cultures progressed, while the levels of cytokines IL-9, IL-17, IL-27, IL-1ra, IL-1B, SDF-1 and TNF-α showed differences as dependent on culture-stages as a time dependent manner.

3D aggregates and 3DRs progressed through cultures. The levels of IL-17, IL-27 and TNF-α showed a decrease by 3DRs while the cytokines IL-1β and SDF-1 showed increased levels (Figure 2). The cytokines TNF-α, IL-1β, IL-1ra, IL-10, IL-6 and IL-9 were seen to be elevated in the 3D as compared to their 2D counterparts. It is known that these cytokines have specific functions for the progression, invasive potential and similar features of cancers[5].

Cancer cell lines are now increasingly being cultured as 3D cultures for a variety of applications. The extension of the culture methodology from 3D to 3DRs as described in this study will provide for enhancing the utility of cells, especially to understand complex cellular processes.

Conflicts of interests: The authors declare no conflicts of interests, financial or otherwise.

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