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Scoring System for Analyzing Vessel Development in Embryoid Bodies

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Abstract

Embryoid bodies (EB) derived from pluripotent embryonic stem cells (ES) are powerful tools for different purposes. One interesting aspect is vessel development and the use for simulating angiostatic effects of drugs. Unfortunately, it is difficult to compare the impacts of different drugs. Current methods allow the description of observations, but quantifying methods are missing.

To overcome that gap, we have developed a score system to transfer microscopic observations into quantifiable values. The score system summarizes different vessel characteristics within few categories and allows the comparison of drug effects in view of time and dosage. We analysed score behaviour with respect to normal vessel development as well as under presence of angiotstatic drugs. The vessel score showed a time-dependent increase under normal conditions while under presence of an angiogenesis-inhibitor the score increase was slowed, and under angiogenesis enhancing conditions the score increase was accelerated.

The presented vessel development score seems to be a helpful tool to transfer microscopic observations in EB vessel development into a quantifiable and comparable value.

Keywords: Embryoid bodies; Angiogenesis; Scoring; Vessel development

Introduction

Vascularization of organs and tissues is carried out by two related, but distinct processes: vasculogenesis and angiogenesis [1]. Vasculogenesis is a process where primitive blood vessels develop from angioblast precursor cells that differentiate and assemble into cord-like vascular structures which further connect into a primary network. Angiogenesis is a process of formation of new blood vessels from pre-existing vessels by sprouting, splitting, and remodelling of vascular network. It is a complex process including a chain of events like endothelial cell activation, growth, migration and capillary morphogenesis [2-5].

The process of angiogenesis requires the involvement of pro- and anti-angiogenic factors. Beside numerous biogenic factors, several modulators such as inhibitors, small molecules and monoclonal antibodies have been characterised that interfere with angiogenesis. Small molecules such as adenosine, 1-butyryl glycerol, prostaglandins E1 and E2 are a few examples also reported for their angiogenic activity [6-9]. Monoclonal antibodies such as bevacizumab and celecoxib and small molecules such as thalidomide are already used extensively in cancer therapy for their anti-angiogenic property [10-12].

Several *in vivo* and *in vitro* models were developed as an attempt to simulate the effects on angiogenesis. *In vivo* and *in vitro* experimental studies show that angiogenic inhibitors might act at different phases of angiogenic cascade such as migration, proliferation, differentiation and three-dimensional organisation of endothelial cells. One powerful tool to simulate angiogenic effects is the use of embryoid bodies (EBs). EBs are spheroidal three-dimensional embryonic tissues grown from pluripotent embryonic stem (ES) cells. They have been shown to differentiate into various cell types of all three germ layers and vessel-like structures effectively improving the diffusion properties of the tissue. Because differentiation of ES cells has been known to recapitulate changes in the embryonic development, all progenic and angiogenic factors that hold essential functions during early embryogenesis are also expected to be involved in the formation of EBs [13].

Up to now microscopic interpretations of angiogenesis by using EBs were focussed on one or only few characteristics of endothelial development [14-16]. To improve the meaningfulness of microscopic observation of vessel maturation, a standardized interpretation method is required which allows the comparison of different experiments. The described scoring system is the first mathematical model which allows the analysis of vessel maturation in EBs in a time-dependent manner.

To prove the method we tested Tyrphastin 1296. Tyrphastin 1296 inhibits the autophosphorilation of PDGFß receptor (PDGFRß) which results in an inhibition of the PDGF pathway [17]. Endothelial sprouting, branching and pruning during angiogenesis depend on PDGFB/PDGFRß signalling [18].

Material and Methods

Cell culture

Mouse blastocyst-derived embryonic stem (ES) cells were established, maintained in culture and differentiated *in vitro* as described before [19,20]. For 2 days hanging drops were kept in 20 µl DMEM supplemented with 20% FCS. Subsequently, EBs were incubated for 3 days in suspension and plated in a 24-multiwell plate on gelatine-coated cover slips for 3, 6, 9 or 12 days in DMEM/15% FCS. After that treatment cells were fixed with 4% paraformaldehyde in PBS for 25 minutes and washed with PBS [14].

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Treatment of EBs

EBs were treated with Testosterone or Tyrphostin 1296 for three days. The time point of adding testosterone was chosen in a way that EB fixation was on day three of incubation. EBs for control were kept untreated.

Immunohistochemistry

Cells were fixed as above and washed several times with 0.05 M TBS and subjected to 0.25% Triton-X 100 and 0.5 M NH_4Cl in 0.05 M TBS for cell membrane perforation. For blocking probes, 5% BSA in TBS was used (1 hour, room temperature). To mark endothelial cells, the antibody rat anti-mouse-PECAM-1 (CD31) was used (1:800, mAb, Pharmingen, San Diego, CA, USA). The relevant secondary antibody was sheep anti-rat Ig biotinylated (1:400, Amersham, LIFE SCIENCE, Little Chalfont, Buckinghamshire, England). This biotinylated antibody was detected with Extravidin conjugated CY 3 (1:1000, Sigma Chemicals, St. Louis, MO, USA; absorption 552 nm, emission 565 nm).

Statistical analysis

All data are presented as mean SD. Data analysis was carried out using Student`s test for unpaired data. Significance was considered at a p value < 0.05.

Results

The aim of the developed score system was to achieve an objective method for quantifying vessel formation and ve s sel maturation in embryoid bodies (EBs). Therefore microscopic o b servations were categorized into seven different properties. P roperties were sub-classified as qualitative (three properties) and quantitative (four properties). Each single property has been a ssigned a score value. The single score values were then added up for the quantitative and qualitative properties respectively. Both achieved values were afterwards calculated as relative values in view of t h e achievable maximum (quantitative: 51 points; qualitative: 80 points). Both were then used as quantitative (Sqn) and qualitative score (Sql). The complete Score was derived as a product of both:

$$Score = (Sqn \bullet Sql)$$

Quantitative Microscopic Properties

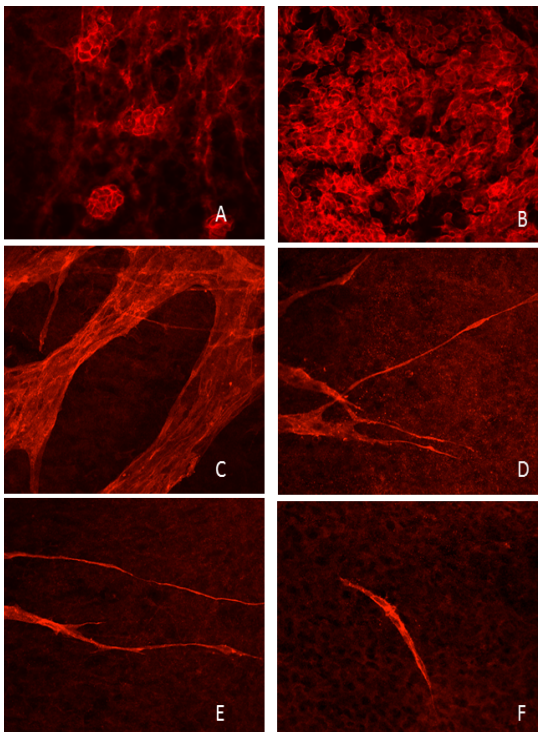
Three different chara cteristics were classified as quantit ative microscopic propert ies. These properties were length, t hickness and the amount of vessels. In case the observed vessels were almost short instead of long, the length score achieved 1 point. In the opposite case (more long then short vessels) the score value with respect to length was 3 (Figure 1) (examples for short and long vessels are shown in Figure 1E and 1F). The same happened with the thickness of the vessels, the only difference being that higher score values were assigned (examples for thin and thick vessels are shown in Figure 1C and 1D). An intermediate group was defined for thickness and length. The amount of vessels was only classified as few or many (Table 1).

Qualitative microscopic properties

Qualitative pro perties are more difficult to objectify. Several microscopic obs ervations, that correlate to certain steps within the vessel maturati on, were described based on their visual appearance (Figure 2). Shr edded vessels appear like short, cutted fragments of vessels (Figure 2C and 2D). If PECAM-positive cells were placed in a direct sequen ce and a short distance. i.e. one after the other, these were referred to as cobble-like (Figure 2E). If the PECAM-positive cell chains showed a plurality of links, this was referred to as network-like (Figure 2A and 2B).

Median effect plot

To describe the relationship between score and vascular development, we used a function analogous to the Hill plot for

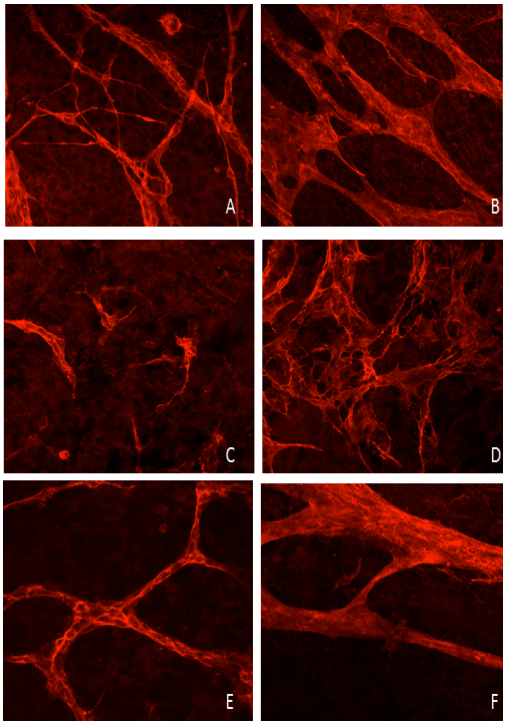


The first pictures illustrate angioblasts in a cluster (A) or plain growing (B). The other pictures illustrate vessels which are mostly thick (C), thin (D), long (E) or short (F). Scale bars: 5 µm.

Figure 1: Confocal microscopy pictures of PECAM stained EBs.

Attributes		Description	Score
Vessel quantity	Length	>70% short	1
		intermediate	2
		>70% long	3
	Thickness	>70% thin	4
		intermediate	8
		>70% thick	16
	Amount	few	16
		many	32
Vessel quality	Shredded	< 5%	0
		5-30%	1
		>30%	2
	Cobble-like	< 5%	0
		5-30%	3
		> 30%	6
	Network-like	< 5%	0
		5-30%	9
		>30%	18
	Mature vessels	< 5%	0
		5-30%	27
		>30%	54

Table 1: Summary of all characteristics and corresponding values.



Picture (A) and (B) show network-like vessels whereas (C) and (D) illustrate shredded vessels. Picture (E) is an example of cobble-like vessel and (F) shows a mature vessel. Scale bars: 5 µm.

Figure 2: Confocal microscopy pictures of PECAM stained EBs.

cooperative systems. By using the median effect plot, which is defined as,

$$\mathrm{Ln}\left(\frac{\mathrm{Score}}{1-\mathrm{Score}}\right)=\mathrm{a}*\mathrm{Ln}(\mathrm{age})+\mathrm{b}$$

the transition of the x-axis shows the point of time, when the half maximum score is reached [21].

Example

In order to validate the efficiency and reliability of the scoring system, microscopic observations and mathematical transformations were carried out as mentioned earlier on normal EBs and on EBs incubated with testosterone. A normal EB yielded a half maximum time of 9.85 days. By incubating the EBs with testosterone, the speed of development was significantly increased. The EBs required only 65% of the time (6.36 days; p=0.049) in comparison to normal EBs which substantiate the angiogenic activity of testosterone. In opposite treating EBs with the PEGF receptor inhibitor Tyrphastin 1296 increase the half maximum time by 50% to 14.9 days (Figure 3).

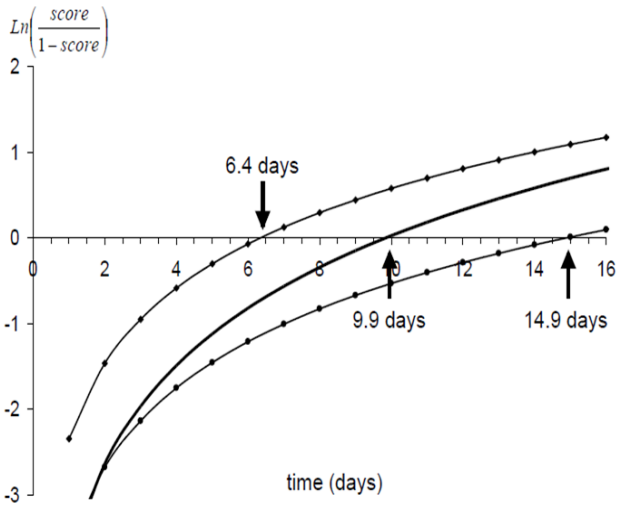
Discussion

Embryonic bodie s can be used to mimic developmental vasculogenesis and angiogenesis *in vitro*. But still current methods do only allow t he description of microscopic observations but not statistical ana lysis. State-of-the-art publications still use descriptions of microscopic observations for the interpretation of drug effects [14-16]. The presen ted scoring system might be useful to overcome that gap and to transfer microscopic observations of vessel development in embryonic bodies into statistical analyzable results. The easiest way to do, might be to use the median effect point of time. This time point is a single va lue which is closely related to vessel development. It is fast to calcul ate, directly comparable and can be used for statistical calculations.

The assay method developed on EB models for angiogenesis requires nothing more than a few critical observations of stained EBs made under confocal microscopy at different points of time which is feasible in any laboratory with basic infrastructure. Various parameters involved in angiogenesis such as the density of angioblasts, length, thickness and also the morphology of the endothelial cells are observed at different time periods. Therefore, this scoring method quantifies morphological parameters into values that are simplified into a score for easy interpretation.

Till date it was unknown that testosterone also has a generalized effect on the endothelium [22]. But through the current scoring method our results indicated that testosterone increased the vessel development. By analysing different parameters as it the case with the scoring model it showed that there is a significant effect on the speed of endothelial development. In only 6.4 days the endothelium reached the median effect point of time in comparison with the control EBs where the speed of development was significantly increased by 35%.

Finally, such a methodology developed incorporating abundant information with respect to different time periods and also under influence of drugs would practically yield results that are very close to reality. Also, the ease of calculation and economical design would aid the portability of the proposed methodology to any clinical setting with only basic infrastructure. Given the above mentioned features, this scoring method can be applied as a potential tool in simulating side effects and assessing novel pro- and anti-angiogenic drugs in vascular therapy effectively. The other areas of interest could be in the area of developing designer steroids for assessing its angiogenic activity.



Normal EB reached a half maximum development score after 9.85 days whereas testosterone treated EBs only have a half maximum time of 6.35 days. EBs treated with Tyrphostin 1296 have a half maximum time of 14.9 days.

Figure 3: Example of vessel development of untreated EBs and treated with testosterone or Tyrphostin 1296.

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